# IMMUNOLOGICAL STUDIES ON EXPERIMENTAL FILARIASIS

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*by*

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This is to certify that this thesis is the original work of Mr. Ramesh Chander Dagai done under my supervision and is suitable for submission for the award of Ph.D. degree in Biochemistry.

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Dedicated to the sweet memory of my revered father Late Shri Nand Lal Bagai.

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## INTRODUCTION AND PLAN OF WORK

Filariasis, first characterized as a communicable disease in humans by Manson and his colleagues nearly 90 years ago, is transmitted from man to man by a blood sucking arthropod and is widely distributed throughout tropical and subtropical territories. In India alone 125 million people are exposed to the risk of filariasis. Recent survey suggests that there are about 12 million microfilarial carriers and another 8 million exhibit disease manifestations in the country.

Research on filariasis, in general, has been largely oriented towards the epidemiological and pathological aspects with less emphasis on the immunology of the disease. More recently, immunologic problems associated with the disease are gaining importance. As regards the diagnosis, the only unequivocal method available today depends on the demonstration of microfilariae in blood or tissues of infected individual. This is at best a slow diagnostic method as microfilariae may appear many months after the infection. Therefore, efforts have been directed by leading workers towards early diagnosis of the infection by immunologic methods. The specificity of the serologic or immunologic reactions in humans resulting from the use of crude extracts or homogenates of animal parasites is doubtful and such data may be misleading due to non-specific reactions (Kagan, 1963a; Schiller, 1967). Information on the antigenic make up of filarial parasites and related helminths is scanty.

There is considerable circumstantial evidence indicating that man develops a well-marked resistance to superinfection (Nelson, 1966). Experimental studies have clearly shown that animals can develop immunity to filarial infections (Bertram, 1966). There is a good presumptive evidence that a proportion of the people can acquire some degree of resistance or immunity, which results in preventing the appearance of microfilariae in the peripheral blood in sufficient numbers for the propagation of the species. The reasons for the absence or disappearance of microfilariae in the peripheral blood of patients with severe elephantiasis, first reported by Manson (1899), are not clear. It is not understood why in cases of established elephantiasis further exposures to infection are of no consequence. In 1960 Scott reviewed 'Immunity to infection with Wuchereria' and concluded that "a certain amount of immunity does exist, but both the degree and nature of this immunity are at present unknown". The paucity of information is due to the fact that experimental studies with human subjects have seldom, for obvious reasons, been practicable. For these reasons progress in understanding the host-parasite relationships in filarial infections has been very slow.

Within recent years it has become evident that specific immunological unresponsiveness can be induced with a variety of antigens before or soon after birth and even in mature animals (Good and Papermaster, 1964). This aspect has received very little attention in connection with parasitic infections although

early infection of the host or prenatal infection in endemic areas might exert a suppressive influence on the immune response of the host.

Today, one of the major problems of the developing countries, like India, is the production of food sufficient in quality as well as quantity for rapidly growing populations. The relationship between famine and pestilence is well known and in recent times experimental studies have shown that nutritional deficiency can decrease resistance to some infections. The importance of the problem of malnutrition and parasitic infections was brought out by Chandler (1957) in his discussion of the interrelations between nutrition and infectious diseases.

Attempts have, therefore, been made in this investigation to throw some light on the above aspects by working on experimental filariasis induced in albino rats. (The advantage for using rodent filariasis is that the infection with Litomosoides carinii, judged by an examination of the microfilarial content of the peripheral blood, is of shorter duration than that in higher animals. Microfilaremia, in general, reaches a peak level in about 3-4 months after the infection and thereafter falls gradually until the parasites disappear altogether from the peripheral blood. The adult worms, however, remain alive and active in the rat throughout this period and for a long time after the absence of microfilariae thus leading to a condition of latency to infection. Thus immunologic aspects of the disease could be conveniently studied during the entire course of the infection.)

The present investigation was therefore undertaken with a view: (i) to isolate the antigens of filarial parasites such as L. carinii, Dirofilaria immitis, Dirofilaria repens and an intestinal nematode Ascaris lumbricoides and to study the complexity and extent of immunologic identity of these antigens by various immunochemical techniques, (ii) to explore the possibility of immunizing the host by (a) active immunization, (b) passive immunization, and (c) by the use of irradiated larvae, (iii) to elucidate the nature and mechanism(s) of acquired resistance in the host, (iv) to study the susceptibility of rats with latent infection to mite-induced reinfection, (v) to study the immunologic response of rats infected at the neonatal stage, (vi) to determine the effect of protein malnutrition on the course of L.carinii infection, and (vii) to assess the susceptibility of albino mice as an alternative host for L. carinii infection.)

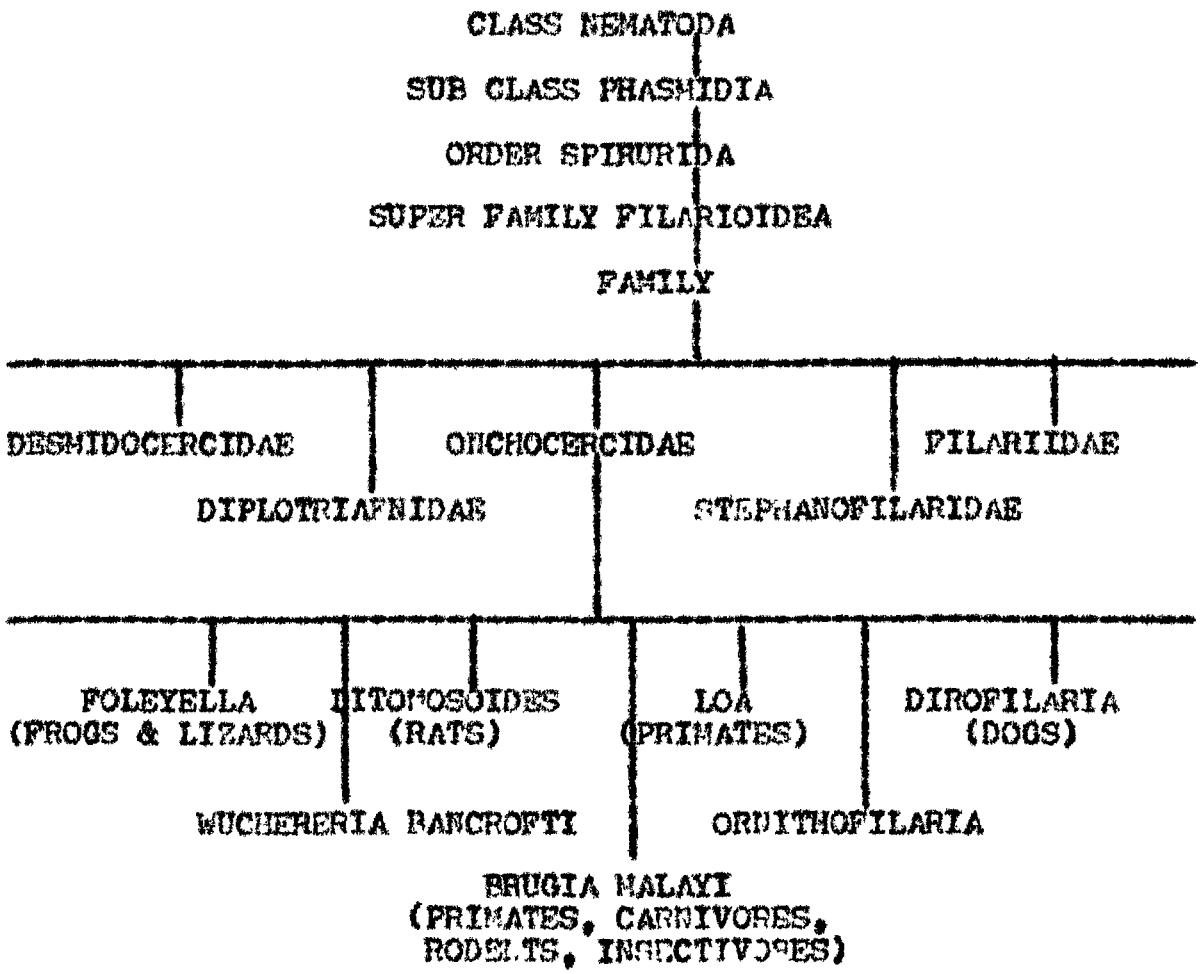
## REVIEW OF LITERATURE

### A. Historical

Filariasis is a very ancient disease. Scientific study on the disease, however, began with the discovery of microfilariae in the hydrocele fluid of a patient in Cuba by Demarquay in 1863. In August, 1866 Wucherer in Brazil, while investigating a suspected case of urinary bilharziasis, found an unknown worm which was the filaria, but this particular record was not published until 1868. Later he discovered microfilariae in chyluria and haematuria. Lewis (1872) discovered microfilariae in the peripheral blood of a Hindu in Calcutta, which he stated could live in the system for months or years without showing any evil consequence. He sent the specimens to Busk for examination, who stated that the worms belonged to the family of Filaridae. Lewis was the first to use the term Filaria Sanguinis hominis for the parasite. Later in 1874 Sansino described microfilariae in the blood and urine in Egyptians. In 1876 Araujo found microfilariae in the blood in Brazil and described the genus as Wuchereria named after Wucherer. Bancroft in 1876 found microfilariae in the blood of a patient in Brisbane. The specimens were sent to Cobbold in London, who recognized the filarial embryos. Bancroft then searched for the adult parasite and announced his first discovery in December, 1876 of an adult filaria worm in a lymphatic abscess from the arm of a Chinaman in Brisbane. The specimens, all

development, which is usually restricted to a definite organ or tissue. During the incubation period they undergo two molts and each larva becomes either an adult male or female worm. The gravid females produce microfilariae which migrate into the blood stream or skin to await an appropriate vector.

Table I



Worms of Clinical Importance

1. Wuchereria bancrofti. The adults live in the lymphatics. The infection is transmitted by mosquitoes which leads to development of chronic lesions such as lymphangitis, elephantiasis, chyluria, hydrocele etc.

2. Brugia (Wuchereria)malayi. This is similar to W. bancrofti.

3. Loa loa. The adults live in the connective tissues and the disease is transmitted by mangrove-fly, chrysops. The lesions caused by this worm include 'Calabar swellings; pruritus and other allergic skin troubles.

4. Onchocerca volvulus. The adults live in fibrous nodules under the skin, and the infection is transmitted by an insect, Simulium. The lesions are due mostly to the microfilariae, which often migrate into the eyes and cause various eye lesions and even blindness.

Worms of Experimental Importance:

1. D. immitis (heartworm). The adults live in the right ventricle of the heart and in the pulmonary artery of the dog. The infection is transmitted by mosquitoes. This worm is of veterinary importance.

2. D. repens. This is similar to D. immitis but the adults are smaller and live subcutaneously in the dog.

3. Dipetalonema witei (blanci, yite), in the jird (Meriones libycus). The adults live under the skin, and ticks (Ornithodorus tartarkovskyi) are vectors of the disease.

4. Dirofilaria uniformis. This infects cotton rabbits and is transmitted by an arthropod, Anopheles quadrimaculatus.

5. L.carinii. Travassos in 1919 discovered a filarial worm from the pleural and peritoneal cavities of the Squirrel, Sciurus sp., from Brazil and named it Filaria carinii (Vaz, 1934). This nematode is identical, according to Vaz 1934, with Filaria patersoni described by Mazza in 1928; with Litomosoides sigmodontis, isolated from the thoracic cavity of the cotton rat (Sigmodon hispidus) by Chandler (1931) and with Micropleura sigmodoni identified by Ochoterena and Caballero in 1932. Vogel and Gabaldon (1932) found the worm in the pericardial and pleural cavities of Mus decumanus and established a new genus Vestibulosetaria and called V. patersoni (Mazza, 1928). But according to Chitwood (1933), the correct generic name should be Litomosoides. Vaz (1934), summing up all the data on this parasite, came to the conclusion that the correct name is Litomosoides carinii (Travassos, 1919). Bell and Brown (1945) have reviewed the synonymy of L. carinii and listed the rodents in which the worm was naturally found.

Laboratory rodents have been investigated as possible experimental hosts by several workers. Culbertson and Rose (1944) introduced this infection in cotton rats as a convenient model for chemotherapeutic experiments. The transmission of the infection was established by Williams and Brown (1946) and by Scott and Cross (1946). Since the first report of

this infection in albino rats by Chandler (1931), several workers attempted to establish this infection in the albino rat (Scott & Cross, 1946; Williams & Brown, 1946; Bertram et al, 1946a; Olson, 1959a; Sen & Bhattacharya, 1961) but described it as unsuitable host for this infection. However, Rohde (1959), and Ramakrishnan et al (1960) showed successful transmission and establishment of L. carinii infection in albino rats. It appears that failure to establish the infection in albino rats by earlier workers might be due to strain differences.

Methods of transmission in the laboratory have been described by Bertram et al (1946b) and Bertram (1947). The infection is transmitted by the tropical rat mite, Ornithonyssus (Bdellonyssus, Liponyssus) bacoti. Similar techniques of transmitting Litomosoides have been reported by Hawking and Sewell (1948), Williams (1948), Scott (1948), Wagner (1956) and Erhardt et al (1964).

C. Detection of immunologic response to helminthic infections:

1. Gel diffusion studies

The gel diffusion techniques used most frequently by immunologist and bacteriologist for the past two decades as a tool in antigenic analysis found application, in recent years, for revealing the complexity of parasite antigens. Of the various procedures devised to study the antigen-antibody reactions in gels, the Oudin tube method and the Ouchterlony plate technique have been used extensively. Graber and

Williams (1956) extended the usefulness of the method by combining it with electrophoretic techniques. The more recent modifications of gel diffusion methods as applied to immunological analysis, in general, have been reviewed by Ouchterlony (1962) and Crowle (1961). These reviews, however, rarely mention work on helminth antigens. Kagan (1961) reviewed various studies in which gel diffusion techniques have been used in the qualitative analysis of parasite antigens. The work on isolation and characterization of helminth antigens employed in immunodiagnostic tests has been reviewed by Kent (1963a).

There are several extensive reviews of serologic and immunologic methodology in the diagnosis of some helminthic infections such as trichinosis (Kagan, 1960), schistosomiasis (Anderson & Sadun, 1962; Jachowski & Anderson, 1961; Kagan & Pellegrino, 1961; Kagan & Norman, 1963), filariasis (Kagan, 1963a; Schiller, 1967), ascariasis (Rodriguez-Burgos, 1961; Taffs, 1961a; Taffs, 1963), echinococcosis (Kagan, 1963b; Kagan *et al*, 1960), paragonimiasis (Sadun & Buck, 1960; Sadun *et al*, 1959b, Sawada *et al*, 1964a, b; Yokogawa *et al*, 1962) and clonorchiasis (Sadun *et al*, 1959a). Recent literature is presented by Haley *et al* (1963) in the proceedings of an 'International Panel Workshop' (Immunodiagnosis of helminth infections).

There are numerous reports on the study of parasitic antigens by gel diffusion (Kagan *et al*, 1958; Soulsby *et al*, 1959; Tanner & Gregory, 1961; Hillyer & Frick, 1967; Hillyer

& Ritchie, 1967; Jeska, 1967; Splitter et al, 1967).

Wodehouse (1956) reported, using Oudin's and Ouchterlony's techniques with immunized rabbit serum, that saline extracts of trichinella larvae contain a minimum of 10 antigens. He analyzed changes in larval antigens after heat, acid and proteolytic digestion and also investigated the presence of common antigens in closely related and unrelated species. Kagan and Bergai (1956) found, using infected rabbit sera, 3 bands in larval antigen of Trichinella spiralis prepared by the method of Melcher (1943). Oliver-Gonzalez and Levine (1962) analyzed similar antisera and demonstrated 3 bands with a larval antigen and 2 different bands with an adult worm extract. In immunoelectrophoresis larval antigen showed 6 or 7 bands while adult worm antigen gave 2 bands (Oliver-Gonzalez, 1963).

Soulsby (1967c) has shown that intestinal tissue of A. lumbricoides suum with homologous antiserum produced 14 bands, out of which 8 could be removed by absorption with sheep erythrocytes, which he believed to be Forsmann antigens. Ascaris cuticle contained 9 antigens; a polysaccharide whole worm antigen produced 4 bands; and a saline whole worm extract showed 9 bands. Similar results were reported by Kagan (1957) in his studies on the antigenic complexity of Ascaris tissues. Toxocara antigens were found to cross react with Ascaris whole worm antiserum. Toxocara canis antigen revealed 5 bands and

tissue antigens. Maximum number of bands were found in sera from infected rabbits 18 days postinfection. Sera from these rabbits cross-reacted with crude extract of D. immitis and formed at least 4 bands.

Soulsby and Stewart (1960) reported antigenic variation in sheep infected with Haemonchus contortus and observed that sera of immune sheep produced a standard pattern of 4 bands. With the decrease in the level of immunity, a decrease in the number of bands in the agar gel plate was observed. On reinfection, which led to "self-cure", a return of bands was noted. The bands were found to be the result of an antibody reaction with the moulting fluid of third stage larvae (Soulsby, 1960).

Other parasitic antigens have been assayed for complexity by agar gel methods. Levine (1959) was the first to apply this technique to schistosomes. He investigated the antigenic complexity of Schistosoma mansoni and Schistosomatium douthitti using a variety of antisera prepared in rabbits against the life cycle stages of both the parasites and sera from infected animals, and reported 2-4 bands with egg antigen, 1 band with miracidia, 1-7 bands with infected snail tissue, 2-6 bands with cercariae and 2 or 3 bands with adult worms. There are numerous other reports on the antigen analysis of schistosomes (Smithers 1960, 1962; Ridges &

& Augustin, 1961; Biguet et al 1962; Kent 1963a; Kagan & Norman 1963; Bentz & Sadun, 1965; Kronman, 1966; da Silva & Ferri, 1965a, 1965b; Sadun et al, 1965; Damian, 1966). All these studies showed that schistosomes are highly complex organisms. Capron et al (1967) have recently demonstrated 21-23 antigenic components in adult S. mansoni and Schistosoma haematobium; 19 of these are common.

Common antigens were investigated between 30 helminths (nematodes, trematodes and cestodes) and 17 hosts (intermediate or final, vertebrate or invertebrate) using Ouchterlony gel diffusion technique and immunoelectrophoresis (Capron et al, 1968). It was found that numerous nonspecific cross-reacting antigens are present in the case of cestodes and nematodes but fewer in the trematodes. The sharing of antigens between host and parasite is thought to be due to adaptation to the parasitic condition.

ii. Passive cutaneous anaphylaxis (PCA)

Helminths are known to be very potent in stimulating the formation of reagin-like antibodies (Ogilvie, 1964; Ogilvie et al, 1966; Hsu & Hsu, 1966; Jones & Ogilvie, 1967; Sadun et al, 1966, 1967, 1968). Several workers have recently shown artificial induction of reagins in laboratory animals such as rats (Mota, 1963a; Bineghi & Benacerraf, 1964), rabbits (Zvailfler & Becker, 1966) and mice (Mota & Peixoto, 1966; Mota, 1967). These antibodies have also been termed

mast cell sensitizing (Mota, 1963a), anaphylactic (Binaghi & Benacerraf, 1964), passive cutaneous anaphylactic (Zvaifler & Becker, 1966), and homocytotropic antibodies (Becker & Austen, 1966). A fundamental property of this type of antibody is to sensitize the skin of the species from which the antibody is derived. An extensive discussion on reagins is given in recent reviews by Stanworth (1963), Hogarth-Scott (1967a) and Higashi and Chowdhury (1968).

The common laboratory animals such as guinea pigs, mice and rats are generally used to demonstrate skin sensitizing antibodies by the PCA technique (Ovary, 1964). PCA can be elicited either in heterologous or homologous species depending on the nature of the antibodies. It is now known that electrophoretically slow migrating IgG ($\gamma 2$) antibodies sensitize foreign species. The "homocytotropic antibodies" (Becker & Austen, 1966) can be divided into two types based on duration of tissue fixation following passive sensitization and differences in heat and mercaptan lability. One type of homocytotropic antibody is represented by $\gamma 1$ (Nussenzweig et al, 1964), guinea pig $\gamma 1$ (Ovary et al, 1963), rat IgG (Mota, 1964; Stechschulte et al, 1967), and human IgG (Terr & Bentz, 1966; Fireman et al, 1965). This type is relatively heat and mercaptan stable and can be detected by PCA reactions at 4 hours and not at 48 hours following injection. The other type, recognized in the mouse (Mota & Peixoto, 1966),

rat (Mota, 1964; Binaghi & Benacerraf, 1964), rabbit (Zvaifler & Becker, 1966), and man (Leddy et al, 1962; Terr & Bentz, 1966) is heat labile, mercaptan labile and can be detected by PCA reactions as long as 17 days following passive sensitization of the skin. In man, the skin sensitizing antibody was believed to be mainly in the IgA fraction of immune globulins (Meremans & Vaerman, 1962; Fireman et al, 1963; Terr & Bentz, 1965), but recent studies have shown that the reaginic antibody in hay fever is not associated with any of the previously known human immunoglobulins, but with a new class designated as IgE (Ishizaka & Ishizaka, 1966; Ishizaka et al, 1966, 1967).

D. Immunity to helminthic infections

i. General considerations

The study of immunity in helminthic infections has been reviewed by many workers (Taliaferro, 1929, 1940a, b; Culbertson, 1941; Chandler, 1963b; Soulsby, 1957a, 1960, 1961, 1962; Kagan, 1960; Tromba, 1962; Urquhart, et al, 1962; Sadun, 1965; Gordon, 1967; Taliaferro, 1967; Higashi & Chowdhury, 1968).

The study of immunity to animal parasites began with the discovery of Smith and Kilbourne (1893) on acquired resistance to Texas fever in cattle, which was followed by reports of acquired resistance in other protozoan diseases such as trypanosomiasis, malaria, and coccidiosis. In

helminthic infections most of the early work was in the direction of diagnostic tests, because it was generally believed that helminths, unlike protozoa, were unable to stimulate the host to produce an efficient immunity. In 1916, however, Fusinami demonstrated the development of acquired immunity to Schistosoma japonicum. Later work showed acquired immunity to trichinosis (Ducas, 1921), strongyloidiasis (Standground, 1928), and haemonchiasis (Stoll, 1929).

Current work on helminth immunity may be traced to the original formulation of concepts and hypothesis of four workers, Stoll, Sarles, Taliaferro and Chandler.

Stoll (1928) found that lambs which were lightly infected with H. contortus when placed on a limited pasture area developed high levels of fecal egg counts after some weeks. It was followed by sudden drop in egg counts and subsequently the animals were resistant to reinfection. The phenomenon called "self-cure" by Stoll is of great significance in controlling worm populations in sheep.

Chandler (1932) suggested that immune reactions to helminths might be directed against biochemical targets in the worm, such as enzymes, resulting in impairment of metabolic process of the parasite. The concept was expanded through many angles by Taliaferro and Sarles in a series of papers published from 1938 to 1942. Taliaferro (1940b) emphasized

the advantage of helminth material for the study of fundamental immune mechanisms.

Proposed mechanisms of local and parenteral immunity, described in papers by Chandler (1936a, 1937b, 1938, 1963b, 1966) and by Taliaferro (1934, 1940b), originally with quite opposite views, were later modified to the opinion that concentration of antibodies, rather than specific antibody producing mechanism, is chiefly responsible for the difference between local and parenteral immunity.

Chandler (1935b) observed retarded development, stunted growth, and inhibition of reproduction in super-infection with Nippostrongylus muris and proposed that effective antibodies against helminths were stimulated not by worm tissues, but by worm products. Chandler (1936a, 1937b, 1939), Sarles (1938), and Taliaferro and Sarles (1937) introduced the concept of metabolic products as functional antigens. Some success has been achieved in recent years, by vaccination with "metabolic products" collected from Nippostrongylus (Thorson, 1961), Trichinella (Campbell, 1966), Trypanosoma lewis (Thillet and Chandler, 1957), and A. lumbricoides (Soulsby, 1957b). It was suggested that the term "metabolic products" should be used advisedly for nematode incubation fluids (Weinstein, 1960). Mills and Kent (1965) have shown that T.spiralis larvae, when incubated in immune serum made against the excretions and secretions of the larvae, were less infective to mice.

ii. Active Immunization

Attempts at active immunization against helminths with vaccines made from dead whole worm material, in general, proved futile (Terry, 1968). Vaccination with killed helminths has produced only marginal protection. Non-living material is antigenic but does not contain "functional antigens" which stimulate resistance. There are many reports in the literature showing unsuccessful attempts to produce active immunity with such type of antigenic materials e.g. Avers et al (1946) could not induce immunity to T. spiralis with cooked infected meat. Mayhew (1944, 1949) reported similar results with dead H. contortus material. Stewart (1950a) showed that tissue extracts of H. contortus on injection into sheep produced humoral antibodies, but the animals were not protected against challenge. McFadjean (1953) could not find any evidence of immunity in cotton rats injected with L.carinii adult worm material in Freund's complete adjuvant. Sprent and Chen (1949), Oliver-Gonzalez (1956), and Soulsby (1957a) were unable to protect small laboratory animals against A. lumbricoides by immunization with tissue extracts although some success by subcutaneous injection of embryonated eggs was achieved (Oliver-Gonzalez, 1956; Soulsby, 1957a). Resistance could not be induced in mice vaccinated with eggs, cercariae, or adult worms of S. mansoni (Thompson, 1954; Ritchie et al., 1962; Moore et al., 1963; Sadun, 1963). Smithers (1962b) could not

induce resistance in monkeys with eggs, cercariae, or adult worms of S. mansoni. Crandall and Arean (1966) tried to induce immunity with viable and non-viable A. suum larval and egg preparations in mice and succeeded to some extent when living material was used but none with non-living material. Recently Smithers and Terry (1967) demonstrated a strong resistance to S. mansoni when living adult worms were transplanted into the mesenteric veins of normal monkeys while no immunity was induced by transplanting worms which had been killed by freezing to -78°C immediately before transplant.

The findings of certain workers indicate that immunity could be induced with dead parasite material. Almost always, however, dead vaccines stimulated a milder degree of resistance than living vaccines (Terry, 1968). Ozawa (1930) reported a small degree of protection in dogs by injections of saline suspensions of S. japonicum worms and similar findings were observed by Lin et al (1954). Successful experiments have been reported with homogenates of adult worms, cercariae, and metabolic products of S. japonicum in mice, and rabbits (Kawamura, 1932; Sadun & Lin, 1959). Artificial immunity was produced by repeated intraperitoneal injections in experimental animals with living, heat killed, and dried and powdered trichinella larvae (McCoy, 1936; Geese, 1949). Spindler (1937) succeeded in producing mild degree of resistance against T. spiralis in rats, rabbits

and guinea pigs after feeding them filtrates from the digested flesh of infected animals. Successful attempts to actively immunize animals have been reported for T. spiralis in rabbits (Dorin, 1946) and mice (Shikhobalova, 1953). Immunity was produced in rabbits with a protein fraction from Fasciola hepatica (Urquhart et al, 1954), and a partial protection was achieved with an antigen prepared from whole fluke (Kerr & Petkovitch, 1938).

Silverman and Patterson (1960) found that the injection of fresh or formalin-treated fourth and fifth stage larvae of H. contortus gave 70% protection against challenge. Jarrett et al (1960) described some degree of protection with adjuvant incorporated dead worm vaccine against D. viviparus. Sadun and Bruce (1964) were able to induce resistance in rats to S. mansoni by previously injecting them with worm homogenates. It was found that older rats developed a much greater resistance than did 1-day old rats. Da Silva and Ferri (1968) reported that mortality in mice infected with S. mansoni, which were immunized with adult worms, was 3 times lower than that in a control group of infected mice.

It has been known for a considerable time that antigens associated with living, actively metabolizing parasites are the important ones in inducing good immunity. Thorson (1964) provided direct evidence in support of this idea. He found that protective antibodies from the immune H. muris serum

could be removed by absorption with excretions and secretions of N. muris larvae. He also used (1953) the excretions and secretions as immunizing antigens to produce immunity against N. muris. Similar results with excretion and secretion of T. spiralis larvae have been reported by Chute (1956). Levine and Kagan (1960) were able to protect mice to some extent by vaccinating with metebolic products of S. mansoni.

iii. Passive immunization

Passive transfer of immunity has been the subject of many experiments to demonstrate the antibody basis for resistance against helminth infections (Soulsby, 1962, for review), but the experiments using passive transfer of immune serum have met with different results. Successful transfer of immunity has been reported against T. spiralis in the rat (Culbertson, 1942b; Dorin, 1946; Jones & Ogilvie, 1967), in the mice (Culbertson & Kaplan, 1938; Hendricks, 1953), N. muris in the rat (Sarles & Taliaffero, 1936; Chandler, 1938; Sarles, 1939), Dictyocaulus viviparus in the calf (Jarrett et al, 1955; Rubin & Webber, 1956), Ascaris suum in the guinea pig (Taffs, 1961b), S. japonicum in the mouse (Sadun and Lin, 1959), in dogs and rabbits (Kawamura, 1932). In T. spiralis infection, Culbertson (1943) also found natural passive transfer of resistance from immune mothers to suckling rats. Recently Bruce and Sadun (1964) reported that passive transfer of the blood of monkeys immunized with irradiated cercariae of S. mansoni conferred immunity to rats challenged with S. mansoni.

On the other hand, failure of passive immunization was reported for N. muris in the rat (Chandler, 1934); Ancylostoma caninum in the mouse (Kerr, 1936); T. spiralis in the rat and mouse (McCoy & Bond, 1941; Larsh et al, 1964); S. japonicum in the monkey (Vogel & Minning, 1953); S. mansoni in the rat (Stirewalt & Evans, 1953); S. douthitti in the mouse (Levine, 1959; Levine & Kagan, 1960); L. carinii in the cotton rat (McFadzean, 1953).

Turner (1959) reported that immune serum from sheep which were made resistant by repeated reinfection with larvae of Strongyloides papillosus when given to lambs in an attempt to passively immunize them was ineffective against challenge by 150,000 S. papillosus larvae, while the same immune serum produced, in vitro, oral and anal precipitates around larvae within 4 hours and death within 24 hours. Meisenhelder et al (1960) noted in S. mansoni infection that massive doses of immune monkey blood passively transferred did not confer any immunity. Similarly Weinmann and Hunter (1961) found that immune serum from mice, which had multiple exposure to cercariae of S. mansoni, when given for a long period to infected mice had no effect on worm development, egg production, and distribution of eggs in the tissues. Hunter & Moore (1964) reported that immunity could not be transferred by parabiotic union between isologous normal and infected mice with S. mansoni.

Hsu and Hsu (1966) found that reaginic antibody positive sera from rhesus monkeys immune to S. japonicum, when injected intracutaneously, did not confer any protection against challenge inoculation. Dineen (1967) reviewed the evidence for humoral antibody and the cellular elements in helminth immunity. His own work with the mechanism of resistance of guinea pigs to Trichostrongylus colubriformis shows that humoral antibodies have no effect in inducing resistance to infection as the passive immunization with sera containing reaginic antibody had no effect. Recently Michel (1968) reviewed immunity to helminths associated with the tissues and described that circulating antibody is not closely connected with acquired resistance.

iv. Immunization with irradiated-larvae

The first report on the deleterious effect of ionizing radiation on helminth larvae was by Tyzzer and Honeij in 1916 on T. spiralis. Smorad (1937) demonstrated that larvae of T. spiralis irradiated at 12,000 r developed into adults but failed to reproduce. Similar results were obtained by Levin and Evans (1942) when they exposed the same parasite to 3250 r-3750 r and showed that the resulting adults induce a high degree of resistance to reinfection in the rat.

Zaiman et al (1956) have shown that a single dose of irradiated T. spiralis larvae when given to one of the two parabiotic rats resulted in inducing resistance in both the

rats to reinfection. The infected rat, however, developed a greater resistance than its uninfected parabiotic twin. On the other hand, Nagath and Thompson (1955) could not find any demonstrable immunity in rats exposed to irradiated larvae of T. spiralis. Gould et al (1955) showed that irradiation of T. spiralis larvae at 10,000 r resulted in establishment of sexually sterile adults and subsequent development of immunity depends on the presence of adults in the intestine. Kim (1957) reported that irradiated larvae of T. spiralis induced some degree of immunity in the mice which was due to the pre-adult stage. These findings have been confirmed by Larsh et al (1959) and Zaiman et al (1961).

Jarrett et al (1958a) reported that a single dose of 1,000 X-irradiated larvae of D. viviparus protected 12 out of 15 calves to further exposure under field conditions. Lucker and Vogers (1960), however, did not succeed in protecting cattle against lungworms with a single dose of X-irradiated infective larvae of D. viviparus. Two doses of irradiated larvae stimulated a higher degree of resistance in the host than a single dose (Jarrett et al, 1959b; Cromwell, 1962; Edds et al, 1963). It was found that a dose of 60,000 r produced over-irradiation and was not effective, whereas doses of 20,000 r and 40,000 r would prevent the lung worm larvae from developing to a stage where they would extensively damage the lungs of the host. Neverthless, this level of irradiation did not

destroy their property of conferring immunity on the host. The attenuated larvae reach the mesenteric lymph nodes, exert an antigenic effect and then die without causing disease. A vaccine was produced by irradiating the larvae with X-rays at a dose of 40,000 r commercially in 1959 (Poynter et al, 1960) and since then has been widely used (Jones & Nelson, 1960; Nelson et al, 1961; Engelbrecht, 1961; Edds, 1963). Jarrett and Sharp (1963) suggested that the third and the fourth stage larvae of the lung worm are the most immunogenic.

As regards the other parasites, irradiated vaccines have been shown to be effective in haemonchiasis. H. contortus larvae irradiated at 40,000 r and 60,000 r have been used to give protection against subsequent infections (Jarrett et al, 1958b, 1959a; Rose et al, 1959; Mulligan et al, 1961), and as in the lungworm the use of double vaccination has been found to give better protection (Jarrett et al, 1961). Soon after, Manton et al (1962) showed that in young lambs irradiated larvae of H. contortus did not confer any protection against a challenge inoculation although in older sheep this would confer a powerful protective immunity to reinfection. These young lambs, although immunologically competent at this stage in respect of bacterial and viral vaccines, appeared to be incompetent as far as H. contortus was concerned. These results were later confirmed by Urquhart et al (1963, 1966 a,b), who showed that a dose of 10,000 H. contortus larvae irradiated at 40,000 r when

given orally once or twice to 3 months old black face lambs failed to protect them against subsequent challenge although an identical regimen of vaccination was effective in sheep of the same breed aged 7 months or more. Different techniques of immunisation including the use of Freund's adjuvent with Haemonchus antigen alone or combined with Fasciola antigen, intraperitoneal injections of normal Haemonchus larvae, reduction in the number of irradiated larvae used for a vaccination dose, and substitution of a serial daily challenge exposure for a large single challenge dose did not result in inducing resistance to reinfection with H. contortus in young lambs (Urquhart et al, 1966b). Recently Bitakaramire (1966) reported that the immune response in older sheep is directed against the third and the fourth larval stages of H. contortus.

Miller (1964) found that X-irradiation reduced the infectivity of A. caninum larvae. He reported that subcutaneous vaccination of dogs and pups with X-irradiated larvae at a dose of 40,000 r is very effective in inducing immunity against the challenge infection (Miller, 1965, 1967). The arrest of the irradiated larvae during somatic migration may be responsible for optimal immunity (Miller, 1966).

Katiyar et al (1968) attenuated infective larvae of Nippostrongylus brasiliensis by exposure to ultraviolet rays for 2 hours from a distance of 130 cm. and injected them into

young rats. Only about 0.1 per cent of the irradiated larvae developed into adult worms as compared to 30 per cent of unirradiated larvae and these larvae produced relatively few ova. They found that subcutaneous injection of various doses of these irradiated larvae conferred considerable protection against challenge in rats.

Various other studies have been carried out using irradiated larvae in infections with T. colubriformis in the sheep and guinea pigs (Jarrett et al, 1960a; Gordon et al, 1960; Mulligan et al, 1961), Trichostrongylus axei (Sinha, 1967), Ascaris infection in white mouse (Villella et al, 1960).

Amongst the trematode parasite this procedure has been most intensively applied to the schistosomes and varying degree of protection has been observed in mice and monkeys to S. mansoni and S. japonicum following previous exposure to irradiated cercariae (Villella et al, 1961; Smithers, 1962a; Hsu et al, 1965; Rodke & Sadun, 1963; Sadun, 1963; Sadun et al 1964). Parasitological and histopathological observations indicated that exposure to irradiation interfere with the ability of schistosomes to reach maturity in mice. The death and disintegration of cercariae induce a considerable degree of inflammation, and it has been shown that different dose levels provide contrasting pathological changes (Lichtenberg & Sadun, 1963). Erickson and Caldwell (1965) observed that in mice exposed to S. mansoni cercariae irradiated at 8,000 r- 10,000 there was a significant decrease in the number of

worms maturing from a challenge infection. Smithers (1962a) reported that large numbers of irradiated cercariae were necessary to induce immunity in the monkeys. Further observations by Smithers and Terry (1966, 1967) showed that in the S. mansoni-rhesus monkey system, the major stimulus to immunity is provided by the adult worm rather than a short lived schistosomulum, such as is derived from an irradiated cercariae.

v. Acquired resistance to helminthic infections

a. Role of humoral factors

Several efforts have been made to demonstrate the effects of immune serum on the parasites in vitro and in a number of cases the formation of precipitates at the body openings of the worms has been reported. Sarles and Taliaferro (1936) showed that when larvae of N. brasiliensis were incubated in the serum of an immune rat, precipitates formed around the orifices of the larvae. They found similar precipitates around larvae migrating in resistant hosts. Later Sarles (1937, 1938) demonstrated precipitates around the larvae of N. muris in homologous immune serum. The precipitates were seen at the mouth, anus, excretory pore, cuticle and intestine of the larvae and were formed as a result of antigen-antibody reaction. Since then in vitro formation of precipitates has been demonstrated for a number of nematode parasites (Oliver-Gonzalez, 1940; Hawkins & Cole, 1945; Sadun, 1949; Thorson, 1963 ,

Smith, 1953; Macdonald & Scott, 1963; Jackson, 1959; Turner 1959; Silverman and Patterson 1960; Taffs, 1961c; Douvres, 1962; Dhar & Singh, 1968). It has been suggested that although a particular immune serum may be shown to have a marked effect on a parasite in vitro it does not necessarily follow that it will constitute a hazard to the parasite in its host (Urquhart et al, 1962). Precipitates were observed on larvae of Ascaris when incubated with immune serum (Sadun, 1949; Soulsby, 1957a). It was noted that the third stage larvae were primarily involved in the formation of precipitates in immune serum. Further studies of Soulsby (1961) indicated that larvae of a challenge infection in Ascariasis were affected in the immune animals only at the time of moulting period. The implication seems to be that the various developmental stages vary in their ability for the release of antigens which will operate in a functional capacity, and in their susceptibility to the host response.

The presence of reagin-like antibodies was demonstrated in rats infected with N. brasiliensis, in rats and monkeys infected with S. mansoni and in sheep infected with T. colubriformis (Ogilvie, 1964). Rats when immunized with adult worm extract, with or without Freund's adjuvent did not produce the anaphylactic antibody. Based on the 'self-cure' response occurring soon after the appearance of reagin-like antibody in rats infected with Nippostrongylus Ogilvie (1967) suggested that this type of antibody might be

responsible for immunity to the infection. Further, reagin-like antibodies formed in rats and monkeys infected with S. mansoni when injected into the skin of normal animals prevented the penetration and development of cercariae applied to such treated sites (Ogilvie et al, 1966). However, recent studies by Ogilvie and Jones (1967) did not altogether confirm the protective nature of reaginic antibodies. Hsu and Hsu (1966) reported that reagins are not involved in acquired resistance to S. japonicum since transfer of PCA-antibody positive sera did not protect against a challenge exposure. More recent studies by Hogarth-Scott (1969) indicated that homocytotropic antibody is associated with the "self-cure" reaction in Ostertagai infection in sheep.

### b. Role of cellular factors

The role of cellular elements of the immune response in resistance to metazoan infections has not received the attention that has been focussed upon humoral antibody, and the cellular reactions which occur in infected tissues have been dismissed generally as secondary non-specific phenomena with a primary antibody reaction (Dineen, 1967). Recent studies have shown that cell-mediated immunity plays an important role against some helminth parasites (Lorsh et al, 1964; Wagland & Dineen, 1966; Dineen & Wagland, 1966; Lang, 1967; Miller, 1967; Lorsh, 1967b).

Delayed (cellular) hypersensitivity differs in certain important respects from immediate (humoral) hypersensitivity (Crowle, 1962). The induction period for sensitization is considered to be about the same for both the types (7-10 days; Raffel, 1961) but they differ in the time required to elicit a reaction in the sensitized host in presence of the antigen. The immediate hypersensitivity is apparent within 3-4 hours of challenge with the antigen in the sensitized subject, can be transferred passively with serum containing antibodies and is unaffected by host-irradiation. It is accompanied by increased permeability of blood vessels and oedema and the hypersensitivity state can be detected, except the atopic type, by usual serologic techniques. Delayed hypersensitivity, in contrast, exhibits reactions in the sensitized host at least 6 hours after challenge reaching maximum in 24-48 hours and is depressed by host-irradiation when given in suitable doses. It is accompanied by intense cellular infiltration and could be transferred only by cells such as lymphocytes. There is no *in vitro* method to detect the antibodies involved in delayed hypersensitivity. Evidence for the involvement of circulating antibodies having affinity for the cells is lacking (Coe *et al.*, 1966). However, an *in vitro* method of detecting sensitized lymphocytes has recently been developed by David *et al.* (1964). These workers demonstrated the inhibition of migration of peritoneal macrophages collected from sensitized animals which contained some lymphocytes in presence of the antigen.

Serum from the sensitized animal was ineffective in inhibiting the migration of macrophages by the antigen (David et al, 1964; David & Paterson, 1965). It is believed that a migration-inhibitory factor is released by the interaction of antigen with the sensitized lymphocytes which inhibits the migration of macrophages (Bennett & Bloom, 1968; David, 1968).

Wagland and Dineen (1965) and Dineen and Wagland (1966) have shown that immunity to T. colubriformis infection adapted to an isogenic strain of guinea pigs can be transferred by sensitized lymphoid cells. The reduction in worm burden was demonstrated in recipients within a brief period after receiving sensitized cells. Further, it was shown that the fourth stage of the parasite was most vulnerable to the immune response, while the early fifth stage or adult worms were unaffected (Dineen & Wagland, 1966).

Kim (1966a, b) demonstrated delayed hypersensitivity reaction of the tuberculin type in the guinea pig to T. spiralis larval antigens after initial sensitization with the antigen in combination with complete Freund's adjuvant. A 7-day latent period proved optimal for establishing the sensitivity which could be transferred from sensitized donors to normal animals by spleenic cells while no precipitating antibody was detected in the serum (Kim et al, 1967).

Larsh and colleagues (Larsh et al., 1964, 1966; Larsh, 1967a) demonstrated the role of cellular factors in immunity

to adult T. spiralis in mice. Cells taken from peritoneal exudate from infected mice when injected intraperitoneally into normal isogenic mice conferred protection on further challenge (Larsh et al., 1964). However, lymph node cells were ineffective. According to Higashi and Chowdhury (1968) negative results could be due to injection of inadequate number of cells, since the draining mesenteric lymph nodes must have been sensitized to the gastrointestinal parasites.

Lang (1967) in studies on acquired immunity to F. hepatica in white mice reported that delayed hypersensitivity may play a prominent role. In a subsequent study Lang et al. (1967) found protection of mice with an intraperitoneal injection of $2.75 \times 10^6$ peritoneal exudate cells, collected from infected donors, to a challenge infection 21 days later with metacercariae while 20 per cent of the infected controls died. At 40 days after challenge the common bile duct of the experimental animals was found to contain 40 per cent of the worms inoculated, while 75 per cent of the worms were found in controls.

Miller (1967) attempted to transfer immunity by serum and lymphoid cells from A. caninum infected pups to non-infected pups. A significant protection was observed with lymphoid cells alone, more protection with serum alone and the best protection when both were given. The evidence for the delayed hypersensitivity, according to the author, was a striking increase in skin thickness at the site of the challenge when compared to controls.

In a recent review, Larsh (1967b) discussed the role of delayed hypersensitivity in immunity of mice to adult T. spiralis and suggested that this is a common phenomenon in response to tissue-invading helminths. According to Gordon (1967), convincing evidence of delayed allergy to nematodes has not been reported. In the experiments of Larsh et al. (1964) the ability of peritoneal exudate cells but not lymph node cells to transfer immunity indicates a cellular immunity phenomenon rather than delayed allergy. In the opinion of Gordon the phenomenon reported by Larsh et al. appear to be functional immune reactions rather than hypersensitivity. Further, he reported that peritoneal exudates in the mouse are predominantly composed of macrophages on the third day after injection of mineral oil. The phenomenon referred to as cellular immunity, according to the author, is mediated by macrophages, while delayed allergy is ordinarily mediated by cells of the lymphocytic series, and should be transferred by grafts of lymph node cells from sensitized animals.

E. Immunosuppressive agents in the breakdown of resistance

1. Cortisone

Studies on bacterial, viral, fungal and helminthic infections have shown that treatment with cortisone, or related steroids, generally lowers the resistance of animals to latent and induced infections (Kass & Finland, 1953; Thomas, 1953; Schwartzman, 1953; Galliard, 1963, for reviews).

Some interesting reports have appeared showing breakdown of acquired immunity or innate resistance following administration of cortisone for some helminth parasites (Markell & Kerrest, 1955; Ritterson, 1959; Dunsmore, 1961; Briggs, 1963; Urquhart et al., 1965; Wakelin, 1967; Campbell, 1963, 1968).

Weinstein (1953, 1956) found that daily administration of cortisone for a certain period in rats while they were being immunized to N. muris had a marked effect on the inflammatory response of the skins of these animals and as a result most of the larvae successfully penetrated through the skin barrier. Many, however, were trapped due to the cellular response in the lungs, liver and peritoneal membrane, but a greater number matured to the adult stage than in the nontreated immune controls. Weinstein (1956) observed that acquisition of immunity to N. muris was partially suppressed by cortisone, but the same level of cortisone had no effect on immunity already acquired.

Cortisone enhanced the susceptibility of mice to T. spiralis infection (Stonner & Godwin, 1953; Coker, 1956) reduced eosinophilia (Polley et al., 1954), and lowered the acquired immunity of the host (Stoner & Godwin, 1954). Coker (1956a) demonstrated that the cortisone treatment could suppress the cellular response in mice infected with T. spiralis and prevents the development of immunity in normal hosts. Moreover, Coker (1956b) showed that daily administration of cortisone completely inhibited the cellular response of the

host against the invasion of larvae in the muscles.

In T. spiralis infection in the mouse, Markell and Lewis (1957) showed that cortisone treatment resulted in the development of large numbers of larvae in the musculature on reinfection than in the controls. Circulating antibodies in high titer could be demonstrated in infected rats treated with cortisone for 30 days and yet there was no marked resistance to the infection. They suggested that the immune response operative in this infection is due to "tissue hypersensitivity" of the delayed type. The administration of cortisone to infected rats prolonged the intestinal phase of T. spiralis producing 9.7 times as many larvae (Markell, 1958).

In the abnormal host cortisone not only prevents the early tissue reaction but also establishes certain infections in the host which would otherwise be unsuitable (Cross, 1960; Parker, 1961). It was found that mouse refractory to Taenia taeniaformis became susceptible after treatment with cortisone as a result of inhibition in antibody production (Oliver, 1962). Ritterson and Concannon (1968) have shown that innate resistance of Cricetulus griseus (the chinese hamster) to T. spiralis could be completely broken down by cortisone. Olson (1958a, b) and Briggs (1963) could establish L. carinii infection in white rats only on cortisone treatment. This effect was considered to be due to suppression of the natural resistance of the animal. Some resistance, however, was manifested in cortisone-treated rats by the delay of worm migration to the thorax and the stunting of growth and development of the worms.

Recent studies by Ogilvie (1965) showed that previously acquired resistance to N. brasiliensis was suppressed by daily administration of prednisolone or betamethasone. A second infection migrating in drug-treated rats was neither delayed nor inhibited in its development. Moreover, expulsion of the worms from the intestine was inhibited completely and eggs were produced as in the controls. Britov (1965) studied the effect of cortisone and adrenocorticotropic hormone (ACTH) on the intensity of trichinelliasis in dogs and rabbits and found that cortisone, given in the acute stage, increased the intensity of infection 4-fold and inhibited immunogenesis. ACTH had no marked effect on infection intensity or on immunogenesis. Pawlowski (1967 a,b,c) found that adrenalectomy resulted in a rapid expulsion of intestinal T. spiralis from rats. Cortisone treatment completely stopped the expulsion of the worms from the intestines of normal as well as adrenalectomized rats.

A few reports have appeared showing the inability of cortisone in suppressing the resistance. In T. spiralis infection Lord (1958) reported that ACTH minimized loss of weight, decreased mortality, prolonged survival time, and slightly reduced eosinophilia, but left the tissue response unaltered in experimentally infected rats. Treatment with cortisone may either slightly enhance (Coker, 1957) or have no effect (Weinman & Hunter, 1960) on the natural resistance of the mouse to infection with S. mansoni.

The mode of action of cortisone and other adrenal steroids and their effects on infection is not well understood. However, several mechanisms have been suggested: (a) inhibition of inflammation (Menkin, 1960), (b) depression of antibody formation (Gyllenstein, 1962) and (c) alteration in the phagocytic properties of leucocytes and reticuloendothelial cells (Lepper, 1962). In addition it is found that cellular metabolism is altered (Dougherty et al., 1961)

ii. Irradiation

There are very few reports in the literature showing the consequences of host irradiation on the development and/or maintenance of immunity to metazoan parasites, but a number of other reports show that irradiated animals are more susceptible to injected bacteria, viruses and toxins. It has been demonstrated that radiation may influence the immune response of the hosts, thereby showing its value as a tool in studies on immunity (Taliaferro & Taliaffero, 1951; Talmage, 1955; Taliaferro, 1957; Benacerraf, 1960; Petrov, 1960).

Stoner and Hale (1952) reported that whole-body irradiation with Cobalt-60 increased the susceptibility of mice to T. spiralis infection. Dunsmore (1961) has shown that whole-body irradiation of the sheep greatly reduced the number of Ostertagia circumcincta larvae whose development is inhibited. Yerinsky (1961) reported that

X-irradiation caused damage to the haematopoietic system which resulted in interference with the development and maintenance of immunity in mice to T. spiralis. Larsh et al. (1962) studied the histopathology of the intestine of mice immunized to T. spiralis infection and exposed to 460 r of X-rays. The inflammatory response to challenge infection in such mice was small 8 days after the irradiation and was measurably larger at 12 days.

Ahmed (1967) reported unsuccessful attempts to break the natural resistance of guinea pigs, rabbits and white mice to Brugia pahangi or B. malayi infection by irradiation.

The factors influencing the immunological potential of the irradiated hosts are not clear. Intense total-body irradiation causes shrinkage of such organs as the spleen. It also suppresses antibody formation, kills many of the lymphocytes and inhibits the immunological activity of the surviving descendents. Bloom (1948) reported that lymphocytes are exceedingly radiosensitive even after 60 r of total-body X-irradiation. The suppressive effect of whole-body X-irradiation on antibody formation has been recently reviewed by Taliaferro et al. (1964).

F. Resistance to reinfection

Studies on the host-parasite relationship of metazoan parasites have shown that resistance to reinfection may be acquired by the host after a primary infection (Culbertson, 1938, 1941; Taliaferro, 1940a,b, 1948; Chandler, 1953; Soulsby, 1957a, 1958, 1961, 1962).

Acquired resistance to reinfection has been reported in the following infections:

Trichinella - In 1921 Duces reported that rats infected with T. spiralis acquired resistance to reinfection. This work was extended by several investigators and it was found that even a few parasites can stimulate protection to reinfection. (McCoy, 1931b, 1932; Bachman & Molina 1933; Bachman & Oliver-Gonzalez, 1936, Roth, 1939, Culbertson, 1942a; Rappaport & Wells, 1951; Larsh et al., 1956). These aspects have been reviewed elegantly by Kagan (1960).

Nippostrongylus - Rats after recovery from an initial infection with N. muris were shown to be immune to reinfection (Africa, 1931; Schwartz et al., 1931). These results have been confirmed and studied from many angles by Chandler (1932; 1935a, b; 1936a, b; 1937a, b; 1938), Spindler (1933, 1936), Graham (1934), Porter (1935a, b, c,), Sarles (1938, 1939), Sarles & Taliaferro (1936) and Taliaferro & Sarles (1939). A review and theoretical discussion are given on this aspect by Chandler (1937b).

Schistosoma - S. japonicum in mice, rabbits and monkeys (Vogel & Minning, 1953; Sadun & Lin, 1959). However, Hunter et al. (1956) could not confirm these observations, in mice hamsters, and rabbits. Attempts to induce resistance to S. mansoni following a primary infection have been unsuccessful in mice (Oliver & Schneidermann, 1953; Stirewalt, 1953), hamsters, guinea pigs, and rats (Thompson, 1954; Hunter et al., 1961). However, Sadun and Bruce (1964) could induce resistance in rats by previously exposing them to cercariae. Partial resistance was obtained in monkeys (Meleney & Moore, 1954) to this infection, while others reported a greater degree of resistance (Sadun, 1963; Standen & Fuller, 1959; Naimark et al., 1960).

Ascaris - A partial resistance to reinfection in human beings and pigs is reported (Roman, 1939; Morgan, 1931). On the other hand, mice and guinea pigs if previously given small doses of embryonated eggs resisted fatal somatic infections (Wagner, 1938). The immunity acquired, however, is of brief duration (Kerr, 1938).

Litomosoides - In cotton rats infected with L. carinii Macdonald & Scott (1953) reported that a superimposed infection 30 days after a primary infection resulted in retardation of growth and moulting rate of the worms in the reinfected animal as compared to worms of primary infections. Bertram (1953), however, showed that despite great variability

in the lengths of females the worm burden when heavy led to worms being smaller than when worm load is light. Retardation of growth, irrespective of numbers, is certainly also involved (Scott & Macdonald, 1968). It has been shown that 7-day old third stage larvae, fourth stage larvae and young adult worms are much less effective in inducing immune response than equal number of younger third stage larvae (Scott et al., 1956, 1958a; Scott & Macdonald, 1956, 1958). It was concluded that the intensive stimulus for the resistance is a result of the migratory activity of the young infective larvae, the form transmitted by the vector, in reaching the thorax for fourth stage development or due to some functional antigens of the larvae (Scott & Macdonald, 1956, 1958). Further, Scott et al. (1958b) reported that the retardation of growth in a reinfection is not dependent on a continuing presence of the initial immunizing infection. Bertram (1966) suggested than an antigen-antibody mechanism is concerned, apart from numerical or physiological factors, in providing some slight protection for the cotton rat host against unrestricted superinfection with adult worms.

Similarly resistance to reinfection has been demonstrated for T. colubriformis (Taylor, 1934; Stewart & Gordon, 1963), A. caninum (McCoy, 1931b; Otto & Kerr, 1939; Kerr, 1936; Kitamura, 1931). H. contortus (Stoll, 1928,1929;

Ross, 1932; Mayhew, 1941; Stewart, 1950a, b, 1953; Lisenko, 1956), D. viviparus (Michel, 1954, 1955; Rubin & Lucker, 1956; Weber & Lucker, 1959; Jarrett et al., 1959c).

It has been found that a number of factors play a role in the development and maintenance of acquired resistance by the host to reinfection. Resistance is most potent when the host receives a well balanced diet whereas malnutrition, in general, results in increased susceptibility and decreased acquired resistance to parasites (Scrimshaw et al., 1969 for review). According to some workers age of the host influences the degree of resistance to reinfection (Serles, 1929; McCoy, 1931a; Ackert et al., 1935; Manton et al., 1962; Brunsdon, 1962), while others do not attribute much significance to it (Mayhew, 1940, Reppaport, 1949; Larsh & Hendricks, 1949; Stewart & Gordon, 1953).

G. Effect of malnutrition on infection

Malnutrition, in general, causes increased susceptibility and decreased acquired resistance to parasitic infections. There is much evidence that the deficient diet usually appears to benefit helminths and the infection therefore flourishes to a greater degree than in hosts on well balanced diet. Scrimshaw et al. (1959) extensively reviewed the results of many investigators on the interactions of nutrition and infection. There are other excellent reviews in the literature all indicating the close relationship between

diet and host resistance to helminth parasitism (Whitlock, 1949; Chandler, 1953a; Hunter, 1953; Frye, 1955; Roberts, 1957). It has been shown that deficient diet results in the breakdown of the acquired resistance in the following parasites: H. contortus infection in sheep (Ross, 1932; Ross & Gordon, 1933) and in calf (Mayhew, 1945), T. colubriformis in sheep (Stewart & Gordon, 1953), N. brasiliensis in rat when generally deficient diets (Chandler, 1932), a milk diet (Porter, 1935b), diets deficient in vitamin A (Spindler, 1933; Kaneko, 1939; Watt et al., 1943; Riley, 1943), or vitamin C (Matsumori, 1941), or Vitamin B1 & B2 (Watt, 1944) and low protein diet (Wells, 1962, Clarke, 1967, 1968) were fed to the animals.

Foster and Cort (1932a, b, 1935) studied the effect of diet on A. caninum. The poor diet, deficient in animal proteins, vitamins and minerals when fed to dogs resulted in faster development of worms and egg production and at autopsy heavy infestations were found as compared to controls. When the deficient dogs were transferred to normal diet, the egg production dropped with concomitent almost loss of worms. Foster and Cort, thus, found that the acquired resistance disappeared when the dogs were fed on poor diet. It is difficult or impossible to infect dogs with the cat strain of A. caninum or vice versa. They observed that in animals maintained on deficient diet, this specific resistance disappeared and the dogs were susceptible to the cat strain and the cats to the dog strain. Chandler (1932) showed that

N. muris was more easily established in rats on a diet of coarse vegetables and fruits than in rats given better diets. Riedel and Ackert (1950, 1951) studied the effect of protein in the diet on Ascaridia and suggested that the quality as well as quantity of protein is important in limiting the establishment of the worms. Miyasaka (1941) reported in N. muris infection in rats that a high level protein content (22 per cent) resulted in better growth of the animals while the growth of the worms was retarded and their numbers reduced. Donaldson and Otto (1946) found more N. muris in rats given a diet with 9 per cent vegetable protein than in those on a diet with 22 per cent protein, half of which was animal protein. Barakat (1948) reported that lysine-deficient diet affects the ability of rats to develop acquired resistance to N. muris by interfering with antibody production. Seddick (1950) found that animals completely deprived of lysine succumbed to infections with rat-hookworm.

Fraser and Robertson (1933) found fewer H. contortus in lambs given a supplement of skimmed milk than in controls. Ross and Gordon (1933) found difficult to establish an infestation of H. contortus in aged sheep on a low calorie diet which was just sufficient to maintain their weight. On further reducing the protein to 3.1 per cent by giving wheaten straw chaff only, heavy infestation resulted, although the amounts of phosphorus and calcium in the diet

were higher. In H. contortus infection sheep fed on high protein exhibited "self-cure" more rapidly (Gordon, 1948) and showed more resistance to challenge than those on low protein. Laurence et al. (1961) showed that naturally infected Merino lambs succumbed to infections with H. contortus and Oesophagostomum venulosum when fed on a standard diet which was an adequate maintenance ration for normal sheep. Djafar (1962) observed that the pre-patent period of H. contortus was shorter in kids maintained on a protein deficient diet. He found less cellular response to H. contortus, more oedema in the mucosa, submucosa and in the abomasal wall in the kids on low protein and higher pH of their gastric contents than in the non-infected animals.

The mechanism by which generalized malnutrition increases susceptibility to helminths is not known. Reed (1958) suggested that deficient diets might produce their effects by stressing the host with a resulting increase in corticosteroids. The effect of cortisone drugs in causing higher parasite burdens is well known (Galliard, 1963, for review). However, severe dietary deficiency can cause increased susceptibility independant of corticosteroid level (Clarke, 1967). The ability to produce antibodies has been studied in rabbits on a low protein diet (Cannon, 1942), in protein depleted rats (Wissler et al., 1946), and in

chickens on a low vitamin A diet (Leutskaya, 1964). In general, a reduction in antibody titer was observed. In H. contortus infection Djafer (1962) suggested that proteins may play a role in the cellular resistance, gastric mucus composition and tissue regeneration of the abomasum which is important in the defence mechanism of the host against the infection.

H. Immunological unresponsiveness

Young animals are immunologically immature and their response to antigenic stimuli differs markedly from that of adult animals (Good & Papermaster, 1964, for review; Binaghi et al., 1966). There are very few reports in the literature on this aspect in parasitic infections. Immunological unresponsiveness or specific tolerance may permit worms to parasitize host and cause disease.

Immunological immaturity to H. contortus is found in several breeds of British sheep, aged 6 months or less (Manton et al., 1962; Urquhart et al., 1966a); in the East African Merino the period is even longer (Lopez & Urquhart, 1968). Successful attempts to induce immunological unresponsiveness have been described for some parasites: Trichomonas foetus (Kerr & Robertson, 1964), Cysticercus bovis and Mesistocirrus digitatus in calves (Soulsby, 1963) and N. brasiliensis in rats ( Kassai & Aitken, 1967;

Ogilvie & Jones, 1967; Jarrett *et al*., 1966, 1968, 1969).

Kerr and Robertson (1954) reported that large amounts of T. *foetus* antigen given to calves resulted in a suppressed antibody response to the antigen. Studies by Soulsby (1963) with C. *bovis* and M. *digitatus* showed that calves infected at birth exhibited a poor antibody response and an inability to resist second infection at 9 months of age, whereas animals infected at 4-6 months of age responded well to both. He suggested that the difference in reaction of young and mature sheep to infection with trichostrongylid nematodes may be due to immunological unresponsiveness of young sheep.

Jarrett *et al*. (1966) reported that rats infected with N. *brasiliensis* during the first 6 weeks of life do not undergo "self-cure" but remain infected into adult life. Similar results were obtained by Kassai and Aitken (1967) who found that rats infected with N. *brasiliensis* at the neonatal stage were not resistant to the establishment of a second infection and this was not terminated after the normal period. Rats infected 3 times in early life were more resistant to reinfection at 6 weeks of age than rats infected once at birth, but less resistant than adult controls (Ogilvie and Jones, 1967).

Moriarty (1966) reported that in rats, neonatal injections of 10 mg of Echinococcus granulosus-cyst fluid protein induced tolerence to these antigens, which could be terminated by injections of the antigens in Freund's complete adjuvant.

Experiments resulting in failure to produce immunological unresponsiveness in certain parasitic infections have been reported: e.g. T. spiralis in mice (Ewert & Olson, 1960; Olson & Ewert, 1961; Bass & Olson, 1963a). Olson and Hill (1966) could not induce immunological unresponsiveness with extracts of adult T. spiralis injected intracardially into new born mice and challenged 30 days later. Foetal or neonatal mice were injected with various antigens from T. spiralis by various routes and then challenged with infective larvae, but no tolerance was induced (Olson & Ewert, 1961). Bass and Olson (1963b, 1966) reported that neonatal infection of mice with trichinella resulted in more resistance to challenge than littermate controls not infected at birth. New born mice had lower worm burdens, showed no serological differences and rapidly eliminated adult worms from the intestine compared to adult controls.

MATERIALS AND METHODS

Animals - The albino rats and mice of different sex and age obtained from the breeding colony of National Institute of Communicable Diseases were used in the experiments. Rabbits and guinea pigs were purchased through a local contractor.

Parasites - L. carinii and D. immitis parasites maintained at National Institute of Communicable Diseases in rats and dogs respectively were used. D. repens was collected from stray dogs at the Filariasis Training Center, Calicut. Ascaris worms were obtained from stools of a patient.

Mites - The tropical rat mites ( O. bacoti ) were obtained from the colonies maintained at National Institute of Communicable Diseases. The strain was originally obtained through the courtesy of Dr. J. Allen Scott, of University of Texas, USA and Dr. Frank Hawking, of National Institute of Medical Research, Mill Hill, London. The colonies of mites were maintained in glass jars, 7" deep and having a diameter of 3½" or in desiccators 8" in diameter. The open side was covered with nylon cloth with a mesh that would not allow the nymphs or adult mites to escape. This cloth was kept in position by rubber bands. The colony was maintained at a temperature of 75°F and 80 per cent humidity. A 2 or 3-day old baby rat was placed in the containers to feed the mites. The rats were removed after 24 hours by which time they die and the mites leave them.

The mites were infected by feeding on an infected rat, having more than 1,000 microfilariae per 10 cmm in the blood, placed in a cage in the container for 24 hours. Baby rats were kept in the container twice or thrice for their blood meal at an interval of 2-3 days while the microfilariae were in the process of development. A few mites from the colony were withdrawn at daily intervals, starting from the 10th day after infection, to ascertain the development of microfilariae to the infective stage. By the time (10-15 days) the infective larvae develop, the mites were ready to feed on the new host.

The transmission of infection

A number of 20-day old albino rats were exposed to infected mites. Blood from the tail of each animal was examined at weekly intervals for microfilariae, starting from the 40th day after infection. Ten cmm of the blood was taken with a micropipette and spread on a clean slide in a thick smear. The air dried smears were dehaemoglobinized the next day in tap water, fixed by one dip in acid alcohol (2 per cent HCl in methyl alcohol), stained with J.S.B. stain I (Jaswant Singh & Bhattacharji, 1944) for 30 seconds and examined. In rats kept for the development of latency, blood was examined for a further period of 2-3 weeks after the infection became latent.

Thoracic contents of infected rats and rats with latent infection were stained with Giemsa.

Preparation of stains

J.S.B. stain - It was made from the following ingredients:

| | |
|---|---|
| Medicinal methylene blue | 0.5 g |
| Potassium dichromate ($K_2Cr_2O_7$) | 0.5 g |
| Sulphuric acid (1 per cent by volume) | 3 ml |
| Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) | 3.5 g |
| Distilled water | 500 ml |

Methylene blue was dissolved in water and sulphuric acid was added gradually with stirring. After thorough mixing potassium dichromate was added which forms a purple precipitate. When disodium hydrogen phosphate dihydrate is added, the precipitate dissolves after stirring the solution for some time. The solution was then boiled in a flask with a reflux condensor for 1 hour. The stain was ready for use when blue colour of the solution deepens.

Giemsa stain - The stock solution was made from the following ingredients:

| | |
|---|---|
| Powdered stain | 1 g |
| Glycerine (C.P.) | 60 ml |
| Methyl alcohol (absolute, acetone free) | 66 ml |

Glycerine and powdered stain were ground together. When well mixed, the stain was dissolved in glycerine in a water bath at 55-60°C and allowed to cool. It was mixed with methyl alcohol and kept for 2-3 weeks and then filtered for use.

Isolation of parasites

The rats having microfilariae of more than 500 counts per 10 cmm were dissected and blood collected by heart puncture. The pleural cavity was then flushed with sterile normal saline solution and the worms were removed with a blunt hooked needle. For the preparation of antigen, the worms were quickly rinsed with cold saline, care being taken to remove blood clots, blotted on filter papers and weighed. D. immitis and D. repens worms isolated from dogs were treated as above.

For the transplantation experiments the worms were kept in warm saline, carefully counted, and the desired numbers seperated. The sluggishly moving worms were discarded.

Worms were removed from rats or transplanted into rats under ether anesthesia.

Isolation of antigens

Phosphate-buffered saline, 0.02-0.05M and tris buffer and tris-maleate buffer, 0.05M of pH 7.2, 8.4 and 6.0 respectively were used to establish a suiteble medium of defined pH for extracting quantitatively different antigens from L. carinii.

Adult worms weighing about 2.5 g were defatted by grinding with 40 ml of chilled anhydrous ether with a mortar and pestle kept in ice. Ether soluble portion was removed and discarded. The residue was treated similarly with an additional 20 ml of ether. The ether from the residue was completely removed through suction by high vacuum pump. The remaining

residue was ground with 25 ml of phosphate-buffered saline of pH 7.2. The mixture was then centrifuged at 10,000 rpm for 30 minutes in an International refrigerated centrifuge. The clear supernatant fluid was removed. The sediment was again treated with 25 ml of fresh buffered saline and recentrifuged to obtain a second supernatant which was added to the first. The pooled antigen extracts were dialyzed overnight in cold against the same buffered saline with 6 changes. The dialyzed extract was again centrifuged and the clear supernatant was stored at $4^{\circ}C$ until use after addition of merthiolate (1:10,000) as a preservative. Identical procedure was followed when other buffers of different pH were used for the isolation of antigens.

Protein Estimation - Protein content of all antigen preparations was estimated by the Folin-Ciocalteu method as described by Kabat (1961). The method essentially involves addition of 6 ml of 12.5 per cent $Na_2CO_3$ solution and 1 ml of 0.1 per cent $CuSO_4$ solution to 2 ml of the antigen. The solution was mixed and kept for 1 hour at room temperature to ensure maximum color development. One ml of 1→3 Folin reagent ( 1 part freshly diluted with 2 parts of water ) was then added slowly with constant mixing. The color intensity was read after 20-30 minutes in Bosch and Lomb colorimeter at 650 mμ. Crystalline bovine serum albumin (Sigma) was used as standard.

Immunization procedure

Rabbits weighing about 1.7 kg each were immunized with soluble antigens of L. carinii or D. repens suspended in Freund's complete adjuvant (Difco laboratories, Michigan, U.S.A.). Whole worm L. carinii homogenates suspended in the adjuvant were also used for immunization in two rabbits. Antigen-adjuvant mixture was made by mixing equal volumes of protein solution and the adjuvant. Preimmunization bleedings were obtained from all the rabbits as control sera. In case of soluble antigens, a total of 18 mg of proteins per rabbit were given in 2 injections in the toe pads 2 weeks apart. Blood was obtained weekly after the second injection for a period of 6 weeks, and serum isolated.

Serum samples from immunized rabbits were qualitatively tested for antibody titer and sera with high titers were pooled, merthiolated and preserved at -20°C.

Preparation of antilymphocyte serum

Rabbit-anti-rat lymphocyte serum was prepared according to the method of Gray et al (1966). Thymus was excised with scissors under sterile conditions, teased apart and pressed through a stainless steel mesh ( 60 gauge) into saline and the cells collected in a petri dish. The cells were washed three times in saline by centrifugation and then resuspended in a known volume of saline. A 0.2 ml aliquot of cell suspension was withdrawn and placed in 10 ml cylinder, the volume of which was adjusted to 10 ml with 1→3 Ringer's

solution (1 part Ringer's diluted with 2 parts of water) to lyse any red blood cells present. After mixing, a sample was taken in a Neubauer counting chamber and the number of nucleated cells in ten square millimeters was counted. The total number of the cells was then calculated. An appropriate number of cells in saline was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were injected with 0.2 ml of the emulsion into each footpad which corresponded to approximately $10^8$ thymocytes per rabbit. Fifteen days later booster injections of cell suspension ($10^8$ cells per injection) without adjuvant were given intravenously on 3 successive days. Each rabbit thus received a total of $4 \times 10^8$ thymocytes. A week after the last injection the rabbits were bled and thereafter weekly for a period of 6 weeks. The serum was separated, merthiolated and absorbed with washed packed rat erythrocytes in a ratio of 5 vol. of serum to 1 vol. of red cells for removing any antibodies formed against erythrocytes.

Analysis of sera

Serum samples from infected rats and immunized rabbits were analyzed by Ouchterlony (1949) technique of agar gel diffusion with the respective antigens. Noble agar (Difco) in a concentration of 0.8 per cent in merthiolated saline was used for all tests. The double diffusion tests were carried out by pouring 15 ml of melted agar in 3½" petri dishes. Patterns were cut that had

diameters and separations of 5 mm. In micro technique 1.6 ml of 1 per cent agar was poured on to a microscope slide from a syringe. Holes were cut that had diameters of 1.5-2 mm and separations of 7 mm. Precipitin reactions of D. repens and L. carinii antiserum and infected rat serum with antigens from different filarial species and Ascaris were studied by this technique and by the modified method of Björkland (1963) to ascertain common and uncommon antigens between the species. The modification of Björkland involved preparation of agar-serum plates by incorporating in agar infected rat serum or rabbit antiserum to a different parasite antigen than the one under investigation at 25 per cent level. When the antigen of a particular species is placed in the center well, free diffusion of reacting antigens for which antibodies exist in the agar-serum medium is prevented due to excess of antibody around the center well. Only those antigens for which no corresponding antibodies were in the antiserum incorporated in the agar, diffuse and give rise to precipitin bands with the corresponding antibodies in the antisera placed in the surrounding wells. Each antigen was studied by this technique.

Immunoelectrophoresis

Paper electrophoresis apparatus (Electrograph, USA) was adapted to immunoelectrophoresis. The micromethod of Scheidegger (1955) was followed on 6½ x 11½ cm glass plates layered with 1 per cent Difco Noble agar in vernol buffer of pH 8.6 at 2 mm thickness. The same buffer was used in the

electrophoresis chambers. To avoid pattern distortion, lateral troughs were cut before electrophoresis which was subsequently conducted at 20 ma and 250 volts for 6 hours. After the electrophoresis, the agar was removed from the rectangular trough and filled with an appropriate antiserum to be examined. The diffusion of antiserum was allowed to develop at room temperature in a moist environment for 24 hours and the precipitin arcs were recorded. The plates were observed for a further period of 48 hours. Immunoelectrophoresis was conducted with each of the antigens using L.carinii antisera and sera from rats infected with L. carinii.

PCA

PCA was performed in guinea pigs and rats as described by Ovary (1964) with rabbit and rat antibodies using homologous and heterologous antigens. The animals were shaved with an electric clipper one day before the experiment. The method involved injection of 0.1 ml of antiserum intradermally on the clean shaven backs of albino guinea pigs (200-260 g) and male rats (150-200 g). Usually three intradermal injections of antiserum with varying dilutions were made on each side of the dorsal skin with a 26 gauge hypodermic needle. At least 4 different animals were tested for each experiment. The animals were injected 4-72 hours after the passive sensitization of skin with 250 µg of protein antigen in 0.5 per cent Evans blue dye in saline (1.25 ml). The animals were killed 30 minutes after the challenge injection with a blow on the head. The

"blueing reactions" occurring at the site of injection were recorded and photographs of the under-skin taken.

Treatment with mercaptoethanol or heat - Serum samples from infected rats (0.5 ml) were dialyzed against 250 ml of 0.1 M 2-mercaptoethanol for 3 hours at room temperature and then dialyzed against 500 ml of 0.02 M iodoacetamide for 4 hours. The sera were subsequently dialyzed in cold against phosphate-buffered saline with 6 changes. Sera dialyzed against phosphate-buffered saline only were used as controls.

Serum samples from the infected rats were heated at 56°C in a water bath for 2 hours. Unheated samples served as controls.

Agglutination - The agglutinating antibody in infected rat serum and immunized rabbit serum was measured according to kaolin-agglutination method of Takahashi et al. (1961) with the isolated antigens from different species.

Irradiation of infected mites

The infected mites containing 3rd stage larvae were irradiated with a Cobalt-60 source. The mites were taken in specimen bottles, the open sides covered with nylon cloth and tightened with rubber bands. The mites in the bottles were exposed to $\gamma$-irradiation from a Cobalt-60 source maintained at the Indian Agricultural Research Institute, New Delhi. The mites were irradiated at 20, 40, 60 and 80 Kr doses. After irradiation the motility of the mites was checked and

a few mites from each lot were teased apart in saline to determine the activity of infective larvae. The mites and the infective larvae showed no apparent effect on irradiation under the above conditions and continued to be active and motile. The mites were then transferred into desiccators and 20-day old baby rats were exposed overnight, as usual.

Irradiation of rats

The animals were subjected to whole-body irradiation with the same Cobalt-60 source. For this purpose the animals were confined in iron wire cages, which were then irradiated with Cobalt-60. In case of few rats, irradiation was done by exposing them to Philips X-ray superficial therapy unit. The following conditions were adopted for irradiation:

Dose rate..... 2.4 Kr/min.; Distance.....15 cm

Voltage ..... 50 Kv; Current ..... 2 mA

no filter in air, acute dose, temp. 86°F.

The total dose given was 800 r per each rat of 200-250 g weight. The irradiated rats were transplanted with worms 24-72 hours after irradiation. All the animals were given Oxytetracycline hydrochloride in their drinking water (50 mg/l) to control possible bacterial infection. The drug was replaced three times a week.

Cortisone treatment

The cortisone acetate (Rousel, India) was injected at a dose of 50 mg per kg subcutaneously daily for 13 days. The drug treated animals lost weight during treatment and all but few recovered gradually when treatment was withdrawn.

Enumeration of microfilariae in various organs

In order to assess the presence of microfilariae in various organs, the following method was used:

The organs were fixed in formal saline and embedded in paraffin; serial sections were cut with a microtome at 4-5 microns thick and stained with haematoxylin and eosin. The microfilariae were searched in the sections.

For gross examination the tissues were cut into small pieces with scissors and teased apart in saline. The cell suspension was examined under a microscope.

Effect of low protein diet

Thirty albino rats each weighing on the average 61 g were distributed into three groups of 10 rats each, care being taken to have rats of equal weight in different groups. The controls, Group I, were fed standard diet providing 15 per cent protein, the rats in Group II received a synthetic diet of the composition detailed below containing 25 per cent protein and the animals in Group III were fed the same diet as in Group II with the exception that it contained only 5 per cent protein. The feeding was continued for a period of 22 weeks with these diets. The protein of the diet of the last group was replaced by starch.

Rats were caged individually, water and food were offered ad libitum. All rats were weighed once a week.

The composition of the standard diet was as follows: Parts of ingredients per 100 g of diet - Whole wheat flour 72 g, whole milk powder 23 g, calcium carbonate 1 g, dried brewer's yeast 3 g and table salt 1 g.

The composition of the high protein diet was: casein 25 parts; ground nut oil 5 parts; starch amylum 65 parts; salt mixture 4 parts and vitamin mixture 1 part.

Salt mixture - It was made up according to Hegsted et al. (1941). In addition 0.2 g $CoSO_4 \cdot 7H_2O$ and 0.1 g NaF were added to their composition given below:

| | | | |
|---|---|---|---|
| $CaCO_3$ | 600 g | $Fe(C_6H_5O_7)_2 \cdot 6H_2O$ | 65 g |
| $K_2HPO_4$ | 646 g | KI | 1.6 g |
| $CaHPO_4 \cdot 2H_2O$ | 160 g | $MnSO_4 \cdot 4H_2O$ | 10.0 g |
| $MgSO_4 \cdot 7H_2O$ | 244 g | $ZnCl_2$ | 0.6 g |
| NaCL | 335 g | $CuSO_4 \cdot 5H_2O$ | 0.6 g |

Vitamin mixture - The composition of the vitamin mixture is as follows. The following amounts in mg per 100 g diet were taken.

Thiamine hydrochloride 0.6; riboflavin 0.8; niacin 4.0; pyridoxine hydrochloride 0.6; calcium pantothenate 4.0; biotin 0.04; folic acid 0.2; menadione 4.0; inositol 10.0; para amino benzoic acid 10.0; vitamin $B_{12}$ 0.004 and choline chloride 100.0.

In addition to the above vitamins, 100 I.U. of vitamin A, 15 I.U. of vitamin D and 0.6 mg of vitamin E in ground nut oil were fed orally to each rat twice weekly.

During the 4th week it was apparent that the rats on low protein diet were not gaining weight. On 7th week all the rats were transplanted with fixed number of worms into the thoracic cavity. Parasitemia was determined starting from the

3rd day after the operation, twice a week for a period of 2 weeks and thereafter once a week till the study was discontinued.

The experiment was terminated 15 weeks after the worm transplant, at which time all the rats on standard diet and those on high protein diet had been negative for a period of at least 4 weeks.

Isolation of sera

The blood from the animals was collected in centrifuge tubes, precaution being taken to avoid contamination, allowed to clot at room temperature for 1 hr and serum removed after centrifugation at 2000 rpm for ½ hr. in a refrigerated centrifuge. The serum was recentrifuged once more for removing any residual clot. The clean serum thus isolated was merthiolated at 1:10,000 level and stored at -20°C until use. The immune sera were tested separately as well as in pooled lots.

Transplantation of worms into Rats

Rats to which worms were to be transferred to the abdominal cavity were anesthetized with ether, and a small slit made in the abdominal wall under aseptic conditions. The worms were then gathered together, placed on the opening, and pushed through into the abdominal cavity. When transplantation was done into the thoracic cavity; the thorax was opened by two lateral incisions so as to make a narrow opening that remained closed until the time of transfer of worms to prevent

suction pneumothorax. The worms to be implanted were then pushed through the opening by a blunt forceps. The incisions through which the worms were introduced were closed by inserting skin sutures and dusted with sulfathiazole powder. The rats always recovered completely after the operation. On the 3rd day after the transplantation, blood from the tail was examined for microfilariae and thereafter every day until the animals were killed. The rats used as controls in these experiments were of about the same weight as the rats with latent infection.

In case of transplantation from thoracic to abdominal cavity, a few worms were taken from the right thoracic cavity after surgically opening the thorax of the rat with latent infection as detailed above and after the addition of about 1 ml of warm saline to facilitate gentle removal without injuring the underlying lung. The incision was then quickly sutured. The worms were subsequently transplanted into the animal's own abdominal cavity.

Splenectomy - Splenectomy was done under aseptic conditions. Anaesthesia was induced with intraperitoneal nembutal 50 mg per kg body weight supplemented by open ether. An incision was given running parallel to the left costal margin and the spleen was exposed. The blood vessels were tied with a nylon thread and the spleen was removed. The incision was closed by inserting skin sutures and dusted with sulfathiazole powder.

Partial pneumonectomy - Anaesthesia was induced with intraperitoneal nembutal supplemented by open ether. The thorax was opened by two lateral incisions and the sternum was pressed downwards and the underlying lung was taken out with a blunt forceps, care being taken to close the opening during the process to prevent suction pneumothorax. All the lobs were gently gathered together, blood vessels were tied with nylon thread and the lung was excised. The pectoral muscle was released and skin sutures were inserted.

## RESULTS

### A. Antigen analysis of filarial parasites

#### 1. Isolation of antigens

Protein content of antigens of L. carinii extracted with buffers of varying pH is given in Table II. As seen

TABLE II

Protein content of extracts of L. carinii

| Buffer | pH | Protein content, mg/g wet weight of the parasite |
|---|---|---|
| 1. Tris@ -maleate | 6.0 | 16 |
| 2. Phosphate-buffered saline | 7.2 | 44 |
| 3. Tris@ | 8.4 | 17 |

@ Tris-tris hydroxymethyl amino methane

from the Table, phosphate-buffered saline of pH 7.2 appears to be the ideal solvent for quantitative extraction of soluble proteins of L. carinii. The antigens of the extracts isolated with different buffers were examined with rabbit antiserum to L. carinii by gel diffusion. A qualitative comparison of the antigens extracted by the different methods indicated, as shown in fig. 1, that the buffered saline (pH 7.2) extract yielded at least 2 or 3 more antigens from those found in the extracts with other buffers.

# A B S T R A C T

Antigens of the filarial parasites Litomosoides carinii, Dirofilaria immitis, Dirofilaria repens and an intestinal nematode Ascaris lumbricoides have been extracted with phosphate-buffered saline (pH 7.2). The antigens pattern in the parasites has been analyzed by various immunological techniques such as gel diffusion, immunoelectrophoresis, passive cutaneous anaphylaxis and agglutination using sera from rats infected with L. carinii and sera from rabbits immunized with antigens of L. carinii and D. repens suspended in Freund's complete adjuvant.

Rats on infection were found to contain circulating antibodies to the parasites including homocytotropic antibodies of the $\gamma_1$ and reaginic types. These studies reveal that the above parasites share common antigens between themselves in addition to possessing species-specific antigens.

The host-parasite relation in albino rats infected with L. carinii has been investigated in considerable detail with particular reference to the mechanism of the acquired resistance to the infection. The aspects include active and passive immunization, role of humoral and cellular immunity, agents responsible for the development of acquired immunity, effect of protein malnutrition on the infection, nature of the immune response as determined by treatment with

cortisone, whole-body irradiation, and antilymphocyte serum and the behaviour of rats with latent infection to reinfection.

The data on the above aspects suggest that the acquired resistance to the infection is due to cell-mediated immunity.

11. Analysis of antigens using sera from infected rats and immunized rabbits.

Infection of albino rats with L. carinii results in the formation of antibodies to the adult parasites as shown in the gel diffusion test by Ouchterlony procedure (fig. 2). The time of onset of the antibodies in the course of infection has also been investigated. A group of 12 rats was bled (by heart puncture) starting a week after the mite-induced infection. Generally, 0.5-1 ml of blood was taken at a time and serum tested for antibody in micro-gel diffusion plates. In majority of cases anti-adult precipitins were found to be present as early as 7 days after the infection (fig.3). The number of precipitins in the sera appeared to increase with the development of larvae in the thorax.

Results of the antigen analysis of L. carinii by gel diffusion and immunoelectrophoresis are presented in figs. 1 and 4. At least 7 different precipitin bands were detected in L. carinii-rabbit anti-L. carinii plates which were separated further to 12 by immunoelectrophoresis (fig. 4). By agar gel diffusion according to Bjorkland's modification it was found that immunized rabbit serum contained 3 more antibodies than infected rat serum (fig. 5). On immunoelectrophoresis, as well, the presence of more antibodies in rabbit serum than in rat serum was evident

Fig. 1. Agar diffusion patterns of reactions of rabbit antiserum to L. carinii with homologous antigens extracted with buffers of different pH. Center well: Rabbit antiserum to L. carinii; Peripheral wells: 1,4 - pH 7.2; 2,5 - pH 8.4 and 3,6 - pH 6.0 buffer extracts.

Fig. 2. Agar diffusion patterns of reactions of infected rat sera with homologous antigens. Center well: L. carinii antigen; Peripheral wells: serum from different infected rats.

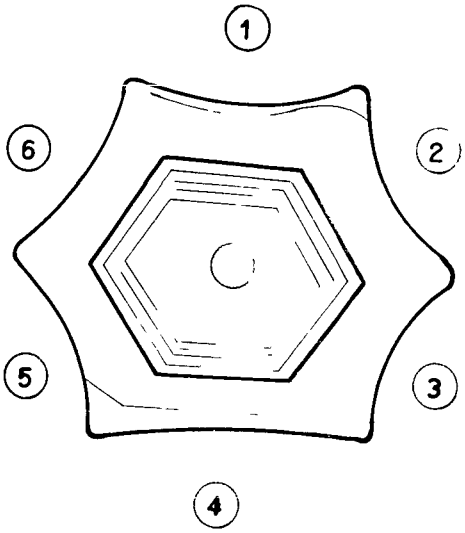



FIG.1

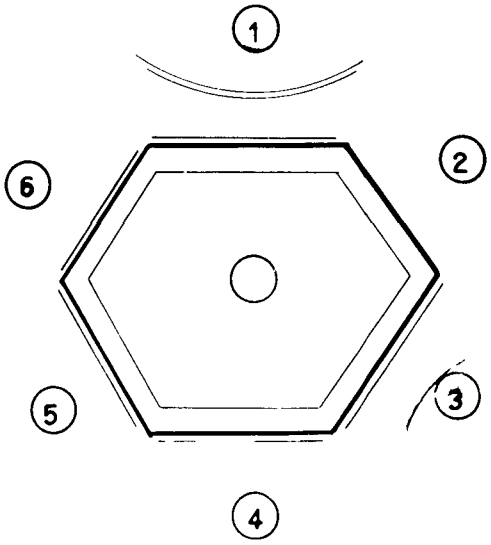



FIG.2.

Fig. 3. Agar diffusion patterns of reactions of infected rat sera with homologous antigen. Center well: L. carinii antigen; Peripheral wells: Serum drawn weekly from infected rats. The number in the wells denote weeks after infection.

Fig. 4. Immunoelectrophoresis of antigens extracted from L. carinii. Diffusion patterns with A, infected rat serum and B, serum from rabbits immunized with L. carinii in rectangular wells.

FIG.3.

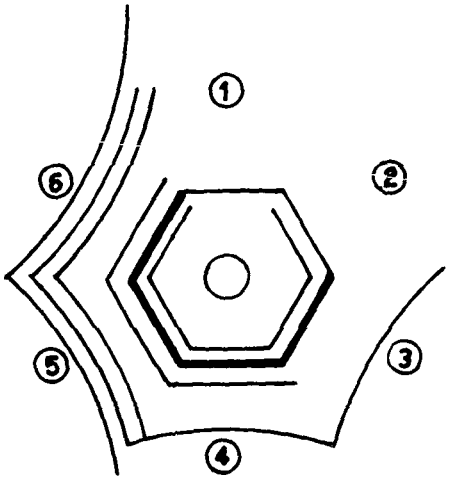
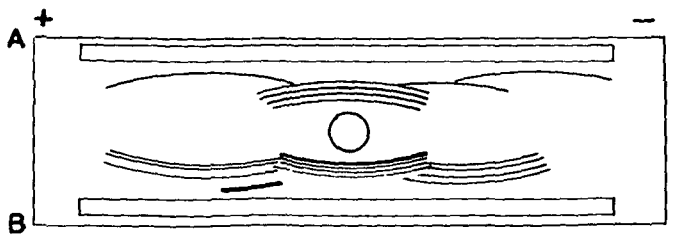



FIG.4.



(fig. 4). It was, however, intriguing to note, as seen in fig. 6, the presence of 2 antibodies in infected rat serum which were not present in immunized rabbit serum.

By the above immunochemical techniques, it was found that D. repens and D. immitis antigens gave rise to 6 identical bands against D. repens antiserum, suggesting that all the antigens present in D. repens were in D. immitis. However, there was considerable difference in the antigenic composition between L. carinii and D. repens. Only 3 of the L. carinii antigens were common with D. repens or D. immitis antigens in double diffusion studies as shown in fig. 7. On immunoelectrophoresis of Ascaris antigens, 4 different antigens were detected that reacted with L. carinii antiserum. Similar studies done with D. repens revealed that it shared 4 antigens with L. carinii (fig. 8). Cross reactions between Ascaris antigens with rat and rabbit serum to L. carinii were also observed in gel diffusion plates. (fig. 7).

PCA studies in guinea pigs using rabbit antiserum to L. carinii revealed cross reactions and hence common antigens between L. carinii and D. repens. Rabbit serum gives a good PCA reaction in guinea pigs against L. carinii antigens but sera from rats infected with L. carinii does not. Infected rat serum, thus, failed to sensitize guinea pigs for PCA when tested with adult L. carinii but PCA could be demonstrated in rats when challenged with

Figs. 5 & 6. Diffusion patterns of reactions of antigens from L. carinii with rabbit antiserum to L. carinii and infected rat serum in plates of agar incorporated with infected rat serum (Fig. 5) and rabbit antiserum (Fig. 6). Well No. 1. - L. carinii antigen; Wells 2,3 - rabbit antiserum and infected rat serum respectively.

FIG.5.

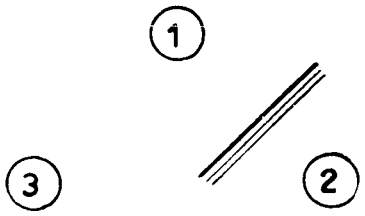



FIG.6.

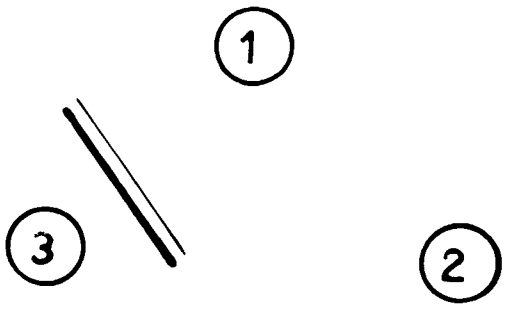


Fig. 7. Agar diffusion patterns of reactions of rabbit antiserum to L. carinii with homologous and heterologous antigens. Center well: rabbit antiserum to L. carinii; Peripheral wells: 1,5 - D. repens; 2,6 - D. immitis; 3 - Ascaris and 4 - L. carinii antigens.

Fig. 8. Immunoelectrophoresis of antigens extracted from D. repens. Diffusion patterns with A, infected rat serum and B, serum from rabbits immunized with L. carinii in rectangular wells.

FIG. 7.

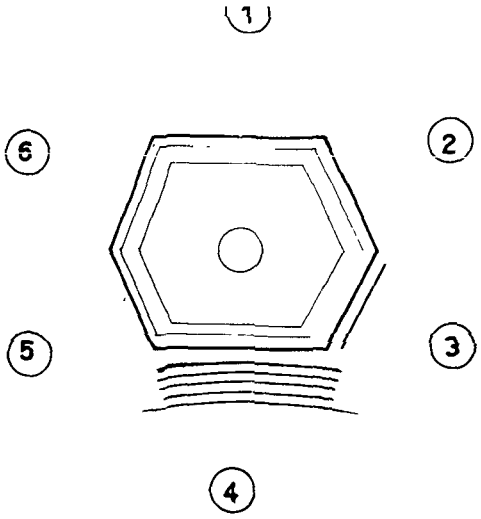
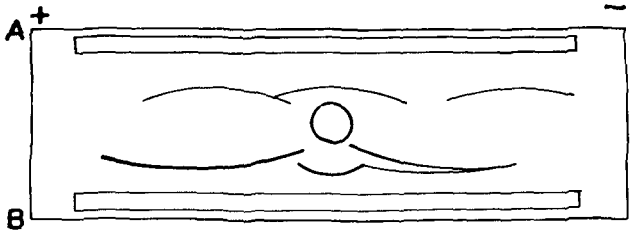



FIG. 8.



antigen at 4 hours and 48 hours after the antiserum injection. This appears to be due to species difference as shown in Plate 1, where rat serum was found to give a good PCA reaction in rats against these antigens. Thus rat on infection with the filarial parasite produces homologous anaphylactic-type (homocytotropic) antibody and a reagin-type of antibody. With rat serum having 6 different antibodies to L. carinii, PCA reaction was positive even at 1:1000 dilution after a latent period of 4 hours after the passive sensitization of skin. Treatment of infected rat serum with mercaptoethanol and iodoacetamide destroyed the reaginic antibody. Heating the serum at 56°C for 2 hours also destroyed the reaginic antibody. However, PCA reaction was positive at an interval of 4 hours.

Agglutination reactions with L. carinii antigens and immunized rabbit and infected rat sera indicate that positive reactions were obtained upto 1:256 dilution and the presence of common antigens were detected between D. repens, D. immitis and Ascaris.

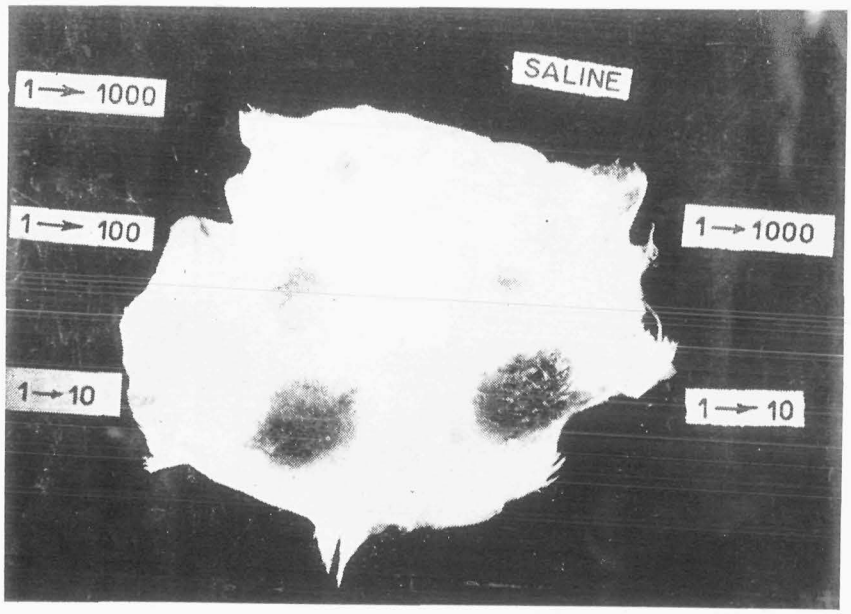
B. Attempts to immunize albino rats against infection with L. carinii.

1. Active immunization

Eight 20-day old rats were injected with 0.3 ml suspension of L. carinii antigen in Freund's complete adjuvent intramuscularly 4 times at an interval of 1 week. Two weeks after the last injection 1 ml of blood was taken from all the

Plate 1. Passive cutaneous anaphylaxis reaction in rat with infected rat serum. Serum dilutions were 1 → 10, 1 → 100 and 1 → 1000 with saline as control.

# PLATE 1.



rats by heart puncture and sera when tested in micro-gel diffusion plates were found to contain precipitins. The animals were then divided into two groups of 4 rats each. The first half were exposed to infected mites while the second half were transplanted with worms into the thoracic cavity. A group of control rats injected with saline were similarly treated. Blood smears for microfilarial examination were taken in the first group after the prepatent period of 40 days while in the second group on the third day after transplantation.

It was found that all the immunized rats transplanted with worms into the thoracic cavity showed microfilaremia count as in the case of controls. Three out of 4 immunized rats developed microfilaremia on infection with mites as in controls (Table III). These observations would indicate that immunization with adult L. carinii extract does not confer any protection to challenge either with transplantation of worms or through natural infection.

TABLE III

Microfilariae in the peripheral blood of four rats immunized with L. carinii antigen and four normal rats after mite-induced infection.

| Rats | Weeks after the incubation period | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Immunized | 0 | 0 | 10 | 37 | 93 | 170 |
| | 0 | 0 | 0 | 18 | 102 | 220 |
| | 0(a) | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 2 | 30 | 125 | 300 |
| Normal | 0 | 0 | 4 | 25 | 160 | 237 |
| | 0 | 0 | 38 | 60 | 185 | 496 |
| | 0 | 0 | 0 | 2 | 7 | 16 |
| | 0 | 0 | 10 | 42 | 90 | 184 |

(a) No adult worms were found in the rat at autopsy. Hence presumed that the rat was not infected.

11. Passive Immunization

Sera from rabbits immunized with whole worm homogenates suspended in Freund's complete adjuvant were used in passive immunization of actively infected rats. Repeated daily 1 ml injections of antisera into the infected rats both intracardially and intraperitoneally for one week did not have any effect on the microfilariae content of the infected animals.

Similar studies were conducted on normal rats weighing about 160 g each. Each rat was injected intraperitoneally with 1 ml of hyperimmune rabbit serum per day for 3 days, and fixed numbers of L. carinii adult worms were transplanted into the thoracic cavity. Subsequently, these rats were injected intracardially with 1 ml of the antiserum for another 4 days. Proper controls with rats receiving normal rabbit serum injections were set up. Parasitemia was observed daily in both the groups. No significant difference in the microfilarial level in rats between the two groups was observed (Table IV).

The above data on the inability of serum that contained antibodies to immunize the rats passively suggest that the circulating antibodies are not protective against infection and have no influence on the microfilariae or adult parasites.

TABLE IV

Effect of injections of hyperimmune rabbit serum on the microfilarial level in the peripheral blood of four normal rats in which worms were transplanted

| Rabbit serum | Days after serum injection | | | |
|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | |
| | 1 | 4 | 7 | 9 |
| Hyper-immune | 2 | 45 | 76 | 187 |
| | 1 | 19 | 94 | 272 |
| | 5 | 14 | 50 | 76 |
| | 0 | 18 | 88 | 168 |
| Normal | 1 | 43 | 79 | 196 |
| | 2 | 17 | @ | @ |
| | 2 | 16 | 42 | 71 |
| | 3 | 33 | 66 | 183 |

@ Counts could not be recorded further owing to the death of the animal.

iii. Immunization with irradiated-infective larvae

There was no significant difference in the motility of larvae irradiated with 20,000 r - 80,000 r when compared to that of non-irradiated larvae. At the highest level of irradiation in the present experiment (80,000 r) both the mites and larvae were found to be alive and active.

At all levels of irradiation, the infective larvae failed to develop and mature to the adult stage, as no immature worms were found in the pleural cavities of the rats when killed and examined 16 days after exposure to irradiated mites. By this time in control rats, which were exposed to non-irradiated infective mites, number of developing worms were found in the thorax. A few animals were then killed and autopsied at different intervals. At the same time blood was taken and sera tested for the presence of precipitins with adult worm antigen by micro-gel diffusion technique. Precipitin bands were noted with sera of all the animals thereby suggesting the successful penetration of the infective larvae into the host.

Three weeks after the initial infection 16 rats, 4 in each group, alongwith 4 uninfected controls were exposed to normal infective mites. Blood was examined for microfilariae 40 days after the infection and thereafter regularly at weekly intervals. The results showed that

the rats originally infected with irradiated larvae were susceptible to reinfection with non-irradiated larvae as revealed by the appearance of microfilariae. Inspection of the data (Table V) reveals that there is no significant difference in the microfilariae level of experimental and control rats, showing thereby that infective larvae irradiated with 20,000 r - 80,000 r are unable to induce any degree of resistance to reinfection.

C. Development of acquired resistance to the infection

The variation in microfilarial content, with time after infection, of the peripheral blood of an infected rat, in general, is as depicted in fig. 9. Microfilariae start appearing in the blood 2 months after infection, reach a peak level in another 2 months, and decline abruptly thereafter till the microfilariae totally disappear from the circulation. The infection at this stage is latent as live adult worms are found in the pleural cavity of the rats eventhough no microfilariae are seen in the peripheral blood.

D. Agents responsible in developing acquired immunity

To find out the role of adult worms in stimulating resistance, a bundle of worms of both sexes was transplanted into the thoracic cavity of 8 normal rats. Blood was taken for microfilarial count one week after the transplentation

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TABLE V

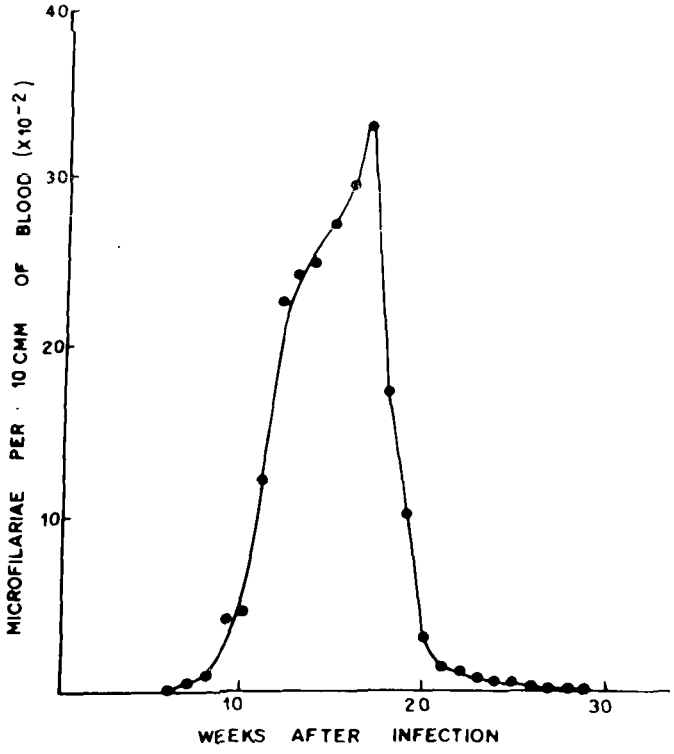
Microfilarial level in the peripheral blood of sixteen rats, which had been initially infected with irradiated mites and four normal rats, after exposure to normal infective mites.

| Level of irradiation | Weeks after the incubation period | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 3 | 4 | 5 | 6 | 7 | 8 |
| 20 Kr | 0@ | 95 | 249 | 869 | 800 | 915 |
| | 0@ | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 68 | 148 | 249 | 266 |
| | 0 | 3 | 49 | 83 | 147 | 279 |
| 40 Kr | 0 | 0 | 0 | 9 | 0 | 0 |
| | 0 | 0 | 62 | 73 | 129 | 200 |
| | 0 | 17 | 82 | 259 | 413 | 680 |
| | 0 | 18 | 169 | 242 | 320 | 360 |
| 60 Kr | 0 | 5 | 64 | 176 | 224 | 244 |
| | 0 | 0 | 0 | 10 | 14 | 26 |
| | 0 | 0 | 46 | 93 | 183 | 370 |
| | 0 | 8 | 92 | 127 | 318 | 560 |
| 80 Kr | 0 | 1 | 21 | 18 | 89 | 103 |
| | 0 | 0 | 21 | 74 | 110 | 170 |
| | 0 | 14 | 86 | 260 | 336 | 460 |
| | 0 | 23 | 73 | 138 | 248 | 274 |
| Normal | 0 | 0 | 33 | 119 | 170 | 280 |
| | 0 | 18 | 133 | 144 | 280 | 400 |
| | 0 | 44 | 148 | 482 | 530 | 746 |
| | 0 | 10 | 72 | 97 | 270 | 300 |

@ No adult worms were found in the rat at autopsy. Hence presumed that the rat was not infected.

Fig. 9. Variation in the microfilarial level of the peripheral blood of an albino rat infected with *L. carinii*.

FIG.9.


40
30
20
10
MICROFILARIAE PER · 10 CMM OF BLOOD (X10⁻²)
10
20
30
WEEKS AFTER INFECTION

and thereafter at regular intervals. The rats usually acquired the latent state in about 10-12 weeks after the worm transfer. The rats were subsequently transplanted with a comparable number of fresh worms alongwith uninfected rats serving as controls. The data showed no microfilariae in the peripheral blood of rats previously transplanted with adult worms whereas controls showed large numbers of microfilariae. It seems from the experiment that resistance can be induced by transplanting adult worms directly into the thoracic cavity of rats without exposing them to mites containing infective larvae. In general, there was a correlation between the microfilariae production of the transferred worms and the degree of resistance induced.

The second experiment was designed to ascertain the role of dead worms, killed immediately after removal by freezing on dry ice before transfer, in inducing resistance. A group of 6 rats was transplanted with 30-50 dead worms of both sexes, another group of 3 rats transplanted with 10-15 live female worms. When the animals of the latter group became latent as shown by the absence of microfilaremia, each animal was challenged with fresh worms. Another group of 5 normal rats similarly transplanted was also included as controls. The data is shown in Table VI. The microfilaremia appeared in rats previously transplanted with dead worms comparable to that observed in controls showing

TABLE VI

Microfilarial level in the peripheral blood of five rats, which had an initial transplant of dead worms and five normal rats, on transplantation with worms, in the thoracic cavity.

| Rats | Days after transplantation | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 1 | 4 | 7 | 10 | 13 | 16 |
| Initially transplanted with dead worms | 2 | 11 | 26 | 52 | 67 | 84 |
| | 6 | 12 | 46 | 40 | 82 | 157 |
| | 4 | 17 | 32 | 29 | 50 | 71 |
| | 16 | 42 | 81 | 103 | 80 | 132 |
| | 7 | 24 | 40 | 72 | 112 | 140 |
| Normal | 4 | 21 | 35 | 64 | 61 | 93 |
| | 8 | 30 | 48 | 169 | 187 | 174 |
| | 6 | 10 | 27 | 34 | 97 | 114 |
| | 9 | 30 | 56 | 89 | 117 | 150 |
| | 3 | 37 | 61 | 90 | 132 | 180 |

thereby that no resistance had developed by prior transfer of dead worms. These experiments indicate that living worms or microfilariae, are responsible for the stimulation of the acquired resistance.

In the next experiment it was attempted to ascertain whether infections in which no microfilariae occurred could give rise to resistance. A group of 6 rats was transplanted with 100 to 150 male worms into the thoracic cavity, another group of 3 rats with 10-15 female worms. After about 10-12 weeks when the rats transplanted with female worms showed no microfilaremia, each animal alongwith 6 normal rats were transplanted with fresh worms. The results (Table VII) show that all the rats, which had received male worms, showed no sign of resistance, suggesting female worms or microfilariae were involved in development of acquired resistence to the infection.

The next experiment was designed to see the role of female worms exhausted of microfilariae in inducing resistance. A bundle of 20-30 female worms was transplanted to uninfected rats, which were then killed within a week after the worm transfer. These worms were recovered and thus passed through a number of uninfected rats for such short periods till they were unable to produce microfilaremia. At this stage 7-12 of these female worms were subsequently transplanted into 2

TABLE VII

Microfilarial level in the peripheral blood of five rats, which had an initial transplant of 100-150 male worms and five normal rats, on transplantation with worms in the thoracic cavity.

| Rats | Days after transplantation | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 1 | 4 | 7 | 10 | 13 | 16 |
| Initially transplanted with male worms | 15 | 25 | 46 | 20 | 27 | 18 |
| | 6 | 24 | 63 | 102 | 183 | 250 |
| | 8 | 22 | 45 | 84 | 110 | 92 |
| | 7 | 78 | 141 | 303 | 580 | 809 |
| | 14 | 32 | 72 | 109 | 170 | 200 |
| Normal | 0 | 5 | 7 | 18 | 13 | 25 |
| | 6 | 10 | 13 | 47 | 63 | 63 |
| | 4 | 29 | 71 | 126 | 177 | 219 |
| | 28 | 62 | 174 | 245 | 396 | 465 |
| | 10 | 24 | 86 | 159 | 219 | 311 |

uninfected rats, a group of 2 rats similarly transplanted with same number of females from infected rats was kept as controls. A week after the development of latency in the control group all the animals were challenged with half the number of female worms originally transplanted. Further evidence that microfilariae play an important part in stimulating acquired resistance was obtained from the results of this experiment as shown by the inability of females exhausted of microfilariae to induce acquired resistance (Table VIII).

TABLE VIII

Microfilarial level in the peripheral blood of two rats, which had an initial transplant of female worms exhausted of microfilariae and two normal rats, on transplantation with worms in the thoracic cavity.

| Rats | Days after transplantation | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 1 | 4 | 7 | 10 | 13 | 16 |
| Initially transplanted with females exhausted of microfilariae | 11 | 53 | 85 | 131 | 200 | 277 |
| | 4 | 16 | 30 | 64 | 113 | 180 |
| Normal | 6 | 26 | 67 | 72 | 109 | 159 |
| | 9 | 41 | 69 | 103 | 194 | 309 |

Role of microfilariae in inducing resistance

Because previous experiments indicated the role of microfilariae in inducing acquired resistance, it was of obvious interest to test the hypothesis by direct experiment. Accordingly, this experiment was designed to determine whether an extended series of injections of microfilariae into normal rats would produce an acquired resistance to further challenge with adult worms. 0.6-1 ml of saline containing uncounted number of microfilariae, isolated from thoracic washings of rats with active infection, were injected into the thoracic cavity of each of three normal rats. The injections were repeated at 3-day intervals. The microfilariae count of the recipients was taken once a week. It was found that about 15-20 such injections containing numerous microfilariae resulted, instead of reaching a very high level of microfilaremia, in a total disappearance of microfilariae in the peripheral blood of the recipients, suggesting the development of a strong acquired resistance. The rats were subsequently transplanted with 4-7 female worms taken from infected rats into the thoracic cavity and a similar number transplanted in normals as controls. The results are presented in Table IX.

It can be seen from the table that rats, which had received multiple injections of living microfilariae, did not show any microfilariae on further transplant with worms, showing clearly that in a natural infection microfilariae

TABLE IX

Microfilarial level in the peripheral blood of two rats, which had received multiple injections of living microfilariae and subsequently cleared all the microfilariae and two normal rats, on transplantation with worms in the thoracic cavity.

| Rats | Days after transplantation | | | | | |
|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | |
| | 1 | 4 | 7 | 10 | 13 | 16 |
| Immunized with living microfilariae | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 |
| Normal | 4 | 9 | 19 | 55 | 47 | 56 |
| | 2 | 6 | 27 | 37 | 34 | 69 |

are the immunogenic agents responsible for the development of a high degree of acquired immunity to the infection.

Transplantation of worms into rats with declining infection

This experiment was designed to determine at what stage of infection immunity to further challenge develops. Accordingly, 6 rats were transplanted with adult worms at different periods in the declining phase after reaching the peak microfilaremia. It was found that the results were variable, some rats were resistant, as the microfilaremia in these rats continued to decline after the worm transplant.

while others were not (Table X). It is concluded that the acquired immunity will be most marked when there is complete disappearance of microfilariae from the peripheral blood. However, it was found that in some rats transplantation of worms resulted in hastening the development of latency.

TABLE X

Microfilarial level in the peripheral blood of six rats with declining infection and six normal rats on transplantation of worms in the thoracic cavity.

| Rats | Peak microfilaremia | Microfilariae count before transplantation | Days after transplantation | | | | |
|---|---|---|---|---|---|---|---|
| | | | Microfilariae per 10 cmm of blood | | | | |
| | | | 1 | 3 | 6 | 9 | 16 |
| Infected | 2248 | 256 | 817 | 236 | 1007 | 1235 | 1090 |
| | 1169 | 47 | 103 | 71 | 16 | 0 | 0 |
| | 1460 | 16 | 15 | 9 | 4 | 0 | 0 |
| | 875 | 9 | 7 | 47 | 90 | 82 | 75 |
| | 1200 | 2 | 9 | 3 | 3 | 4 | 0 |
| | 3342 | 78 | 89 | 65 | 46 | 22 | 0 |
| Normal | | | 7 | 58 | 162 | 332 | 665 |
| | | | 6 | 10 | 59 | 127 | 274 |
| | | | 19 | 43 | 185 | 217 | 366 |
| | | | 3 | 28 | 32 | 40 | 66 |
| | | | 30 | 55 | 53 | 166 | 451 |
| | | | 4 | 25 | 70 | 108 | 245 |

E. Role of circulating antibodies in acquired resistance

As described in the previous section albino rats on infection with L. carinii produce circulating antibodies including homologous anaphylactic and reaginic types. It is not known whether these antibodies have any role in the development of latent infection. Ramakrishnan et al. (1962) suggested, from their experiments on transplantation of parasites from rats with latent infection to the abdominal cavities of normal rats, that immunologic factors present in the circulating blood of rats with latent infection destroyed the microfilariae, thereby causing latency. The appearance of microfilariae in normal rats when the worms from a rat with latent infection were transplanted into the abdominal cavity was confirmed by the results of the present study (Table XI). If the circulating

TABLE XI

Microfilarial level in the peripheral blood of eight normal rats, in which worms collected from rats with latent infection, were transplanted in the abdominal cavity.

| Days after transplantation | | | | |
|---|---|---|---|---|
| Microfilariae per 10 cmm of peripheral blood | | | | |
| 1 | 4 | 7 | 10 | 12 |
| 0 | 4 | 40 | 66 | 103 |
| 1 | 12 | 71 | 117 | 228 |
| 2 | 30 | 64 | 75 | 130 |
| 0 | 1 | 7 | 26 | 37 |
| 0 | 3 | 21 | 40 | 74 |
| 7 | 49 | 86 | 169 | 307 |
| 0 | 2 | 7 | 13 | 26 |
| 8 | 34 | 142 | 179 | 237 |

antibodies were responsible for latency, no microfilaremia would be expected in rats with latent infection when active female worms are transplanted into them. To test this hypothesis, one half of the worms containing uncounted numbers of males and females from actively infected rats were transplanted into the abdominal cavity of rats with latent infection while the other half were introduced in uninfected controls. The results, however, revealed that microfilariae do appear in rats with latent infection eventhough circulating antibodies to adult parasites were present in their blood. Further, it was found that the transplantation of even one female worm was sufficient to result in microfilaremia in certain rats with latent infection, and it was observed that adult worms recovered from the thoracic cavity of such rats with latent infection when transferred into their own abdominal cavity resulted in the appearance of microfilariae in the peripheral blood. However, when the observations were continued in such transplanted rats it was found that the microfilaremia declined and disappeared quicker than similarly transplanted uninfected controls(Table XII). This is expected as seen in fig. 9 where the circulating microfilariae do disappear in rats fast at the time of development of latent infection.

In the next experiment, suspension of microfilariae, collected from the pleural washings of rats recently transplanted with worms, were incubated with sera from rats

TABLE XII

Microfilarial level in the peripheral blood of ten rats with latent infection and ten normal rats, in which worms were transplanted in the abdominal cavity.

| Rats | Days after transplantation | | | | |
|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| With latent infection | 2 | 0 | 0 | 0 | 0 |
| | 20 | 85 | 23 | 5 | 0 |
| | 3 | 22 | 9 | 0 | 0 |
| | 1 | 0 | 0 | 0 | 0 |
| | 9 | 12 | 8 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| | 45 | 180 | 60 | 3 | 0 |
| | 0 | 1 | 0 | 0 | 0 |
| | 24 | 73 | 4 | 0 | 0 |
| | 3 | 2 | 0 | 0 | 0 |
| Normal | 3 | 7 | 20 | 35 | 69 |
| | 16 | 70 | 137 | 266 | 357 |
| | 48 | 75 | 157 | 208 | 287 |
| | 4 | 28 | 128 | 160 | 223 |
| | 13 | 29 | 36 | 60 | 98 |
| | 43 | 68 | 230 | 304 | 460 |
| | 40 | 232 | 393 | 683 | 817 |
| | 36 | 62 | 80 | 103 | 168 |
| | 30 | 66 | 160 | 213 | 295 |
| | 10 | 87 | 200 | 280 | 376 |

with latent infection at 37°C for 24 hours to see the effect of circulating antibodies on microfilariae in vitro. The microfilariae were examined at intervals during the incubation period. No significant difference was found in the activity of microfilariae thus incubated with the sera containing antibodies. Similar observation was made when the microfilariae were incubated with sera from immunized rabbits containing antibodies to the adult parasites at high titer. The lack of any apparent effect on the time of survival upto a period of 24 hours, and the absence of any agglutination of the microfilariae in the rabbit immune serum or serum from rat with latent infection suggest that circulating antibody may not be involved in the development of acquired resistance.

The sera from rats with latent infection containing the reaginic antibodies when injected into infected rats had no effect on the course of infection. RQ of the adult worms remained unchanged in presence of serum from rat with latent infection or immunized rabbit serum as revealed by Warburg respirometer. These studies confirmed that humoral antibodies have little role in the development of latency to infection.

Effect of the blood of a rat with latent infection on the development of microfilariae in the vector.

A colony of hungry mites infected with microfilariae was allowed to feed 2 days after infection on a rat with latent infection to see whether microfilariae in the

intermediate host after coming into contact with humoral antibodies were still capable of developing to mature infective larvae. The control mites were fed on a 3-day old baby rat, as usual. A few mites were dissected the next day to see the condition of the developing microfilariae. It was found that the microfilariae were alive and were in the process of development. The mites were again fed twice on the rat with latent infection at an interval of 2-3 days and were examined 3 days after their last blood meal. The mites were found to contain active mature infective larvae.

The results show that the blood of a rat with latent infection does not prevent the development of microfilariae.

F. Role of cellular factors in acquired resistance

The pleural cavity of the rat has been known to be the natural habitat of adult L. carinii. Singh and Raghavan (1962) observed, contrary to the findings of Rohde (1959), that the right thoracic cavity of the rat was the preferred site of adult L. carinii. This observation was confirmed during the course of the present experiments. It was thought that the constant presence of adult worms in the thoracic cavity might have resulted during the course of infection in some form of tissue response whereby microfilariae produced by the living, active worms could not penetrate or have been agglutinated during the process

of penetration of lung capillaries. To test this hypothesis, one-half of the worms from rats with active infection were transplanted directly into the right thoracic cavities of rats with latent infection, the other half into the right thoracic cavities of normal rats serving as controls. It was observed (Table XIII) that no microfilariae appeared in rats with latent infection except in insignificant numbers in a few, while large numbers appeared in the peripheral blood of normal animals.

The extent of resistance to infection appeared to depend on the initial worm burden and hence the severity of infection. Unless the original infection was high as revealed by peak microfilaremia of about 600 per 10 cmm or higher, transplantation of large number of worms into the right thoracic cavity of rats with latent infection resulted in the appearance of some microfilariae in the peripheral blood. However, the numbers were consistently and significantly lower than those observed in uninfected controls in which worms had been transplanted.

The absence of microfilariae in the peripheral blood does not seem to be due to the inhibition of production of microfilariae by the adult worms. Saline washings from the thoracic cavity of rats with latent infection were found to contain numerous active microfilariae.

TABLE XIII

Microfilarial level in the peripheral blood of nine rats with latent infection and eight normal rats, in which worms were transplanted in the thoracic cavity

| Rats | Days after transplantation | | | |
|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | |
| | 1 | 4 | 7 | 10 |
| With latent infection | 0 | 0 | 0 | 0 |
| | 1 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| | 0 | 0 | 1 | 0 |
| | 0 | 1 | 0 | 0 |
| | 1 | 3 | 3 | 2 |
| | 0 | 0 | 0 | 0 |
| | 0 | 4 | 1 | 0 |
| | 0 | 0 | 0 | 0 |
| Normal | 5 | - | - | - |
| | 29 | 222 | 488 | 816 |
| | 8 | 89 | 342 | 476 |
| | 1 | 49 | 67 | 103 |
| | 3 | 14 | 33 | 85 |
| | 0 | 28 | - | - |
| | 1 | 11 | 22 | 43 |
| | 3 | 25 | 85 | 191 |

- Counts could not be recorded further owing to the death of the animal.

To examine whether the pleural space of rats with latent infection interfered in the migration of microfilariae to the blood, heparinized blood or saline washings from the pleural cavities of infected rats containing microfilariae were injected into the pleural cavities of normal rats and those with latent infection. Known volumes varying from 1-2 ml of heparinized blood (7,376 microfilariae per 10 cmm), and of pleural washings (2,020 microfilariae per 10 cmm) from rats with heavy infection were used for injections. The slides were made 5 hours after the injection of microfilariae and thereafter daily for 10 days. It is interesting to note (TableXIV) that all the treated rats with latent infection showed none or very low

TABLEXIV

Microfilarial level in rats with latent infection and in normal rats injected with heparinized blood or saline washings of pleura containing microfilariae into the thoracic cavity.

| Rats | Rat No. | Days after injection | | | |
|---|---|---|---|---|---|
| | | Microfilariae per 10 cmm of blood | | | |
| | | 1 | 4 | 7 | 10 |
| With latent infection | 1 | 0 | - | - | - |
| | 2@ | 0 | 0 | 0 | 0 |
| | 3@ | 0 | 1 | - | - |
| Normal | 1 | 169 | 80 | - | - |
| | 2@ | 9 | 60 | 89 | 47 |
| | 3@ | 0 | 46 | 26 | 24 |

@ Saline washings containing microfilariae were injected

- Counts could not be recorded further owing to the death of the animal.

numbers of microfilariae in the peripheral blood, while the controls had high microfilaremia.

The results obtained here suggest that the microfilariae may be destroyed by the sensitized cells in the thoracic area before they get into the blood. This sensitization seems to be a result of repeated exposure of microfilariae to tissues during migration. The acquired resistance appears specific in nature since in the next experiment erythrocytes infected with Plasmodium berghei when injected into the thoracic cavity of rats with latent infection resulted in appearence of P. berghei in the peripheral blood within 4 hours after injection.

In order to study further aspects of this resistance microfilariae were searched in sections of spleen, liver, marrow, lungs, lymph nodes and kidneys. Microfilariae were not found in any of the sections examined. It appears therefore that the microfilariae do not reach the blood but are trapped somewhere. A careful search of the pleural exudate suggested that microfilariae are undergoing destruction in the thorax and the results of the following experiments provided strong evidence that cellular reaction is a feature of the immune mechanism.

The thoracic contents of the rats soon after the development of latency were stained with Giemsa. Intense cellular infiltration was observed in the pleura which

consisted of largely lymphocytes and macrophages and to a certain extent eosinophils, mast cells and giant cells. Most of the active microfilariae were found to be in the center of cluster of cells or attached to lymphocytes and macrophages and occasionally to eosinophils as shown in figs. 10, 11 and 12. In most of the microfilariae the nuclei were found to be disturbed from their regular arrangement. The cells were firmly adhered to the sheath or body of the microfilariae thereby immobilizing them (fig. 12), which eventually led to their destruction (figs. 13, 14 & 15).

1. Effect of splenectomy

a. The effect of splenectomy on rats with latent infection

Four rats with latent infection, 3-5 weeks after the development of latency, were splenectomized. Slides were taken 3 days after the operation and thereafter twice a week for a period of 4 weeks. All the rats gave consistently negative results showing that splenectomy does not interfer in the maintenance of acquired immunity to the infection.

b. The effect of splenectomy at the declining stage of infection

Six rats having microfilariae in declining phase were taken for this experiment. Half of the animals were splenectomized while the other half served as infected controls. It was found that the microfilariae counts continued

Fig. 10 Two small lymphocytes and a macrophage attached to the body and sheath of a microfilaria (X 1100).

Fig. 11 Sheath of a microfilaria attached to a big macrophage (X 1100).

FIG.10.

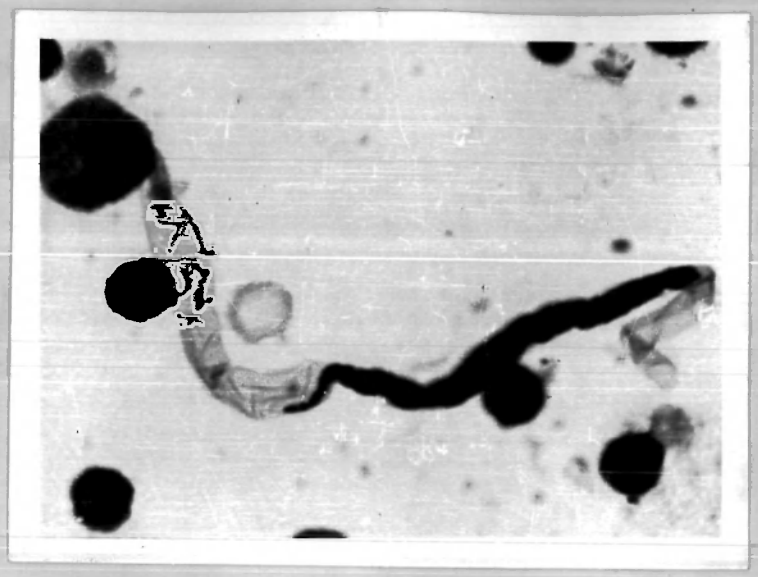


FIG.11.

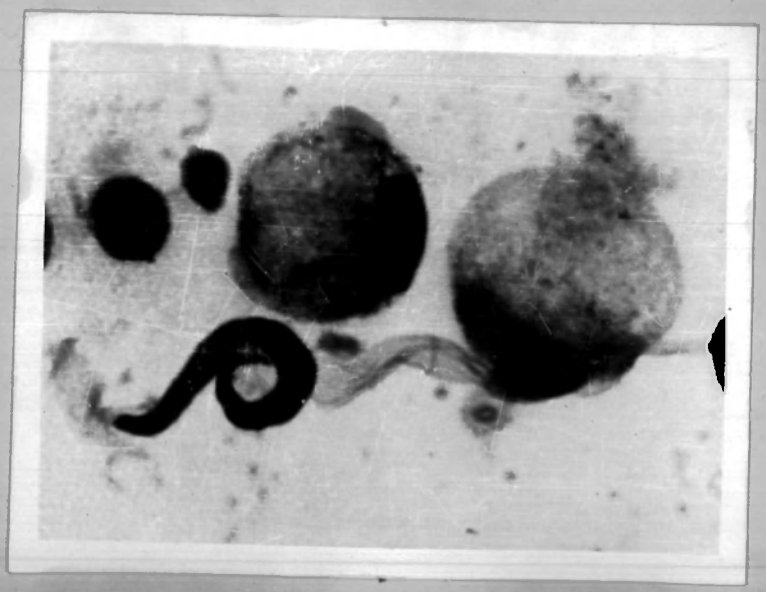


Fig. 12. Microfilaria completely immobilized with lymphocytes and macrophages adhered from both the sides (X 1100).

Fig. 13. Part of a disintegrated microfilaria attached to a big macrophage (X 1100).

FIG.12.

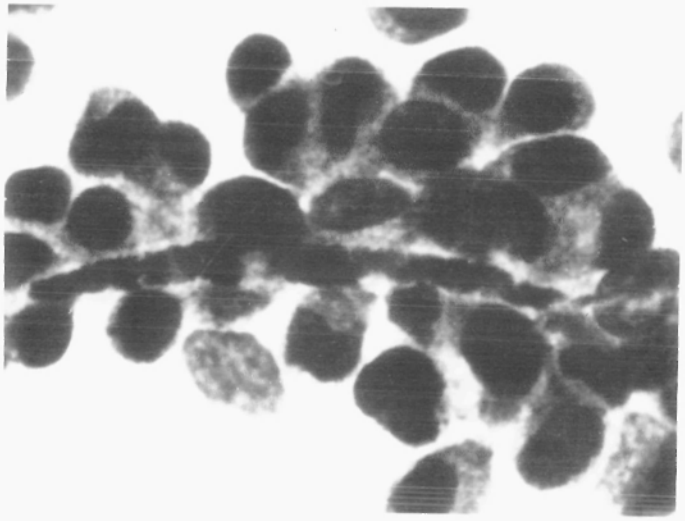
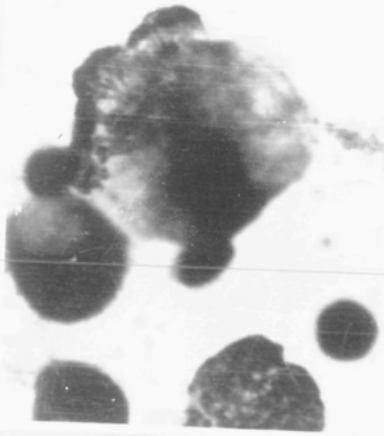


FIG.13.



Figs. 14 & 15. Microfilaria undergoing destruction. Various types of cells are seen in the figs (X 1100).

FIG.14.

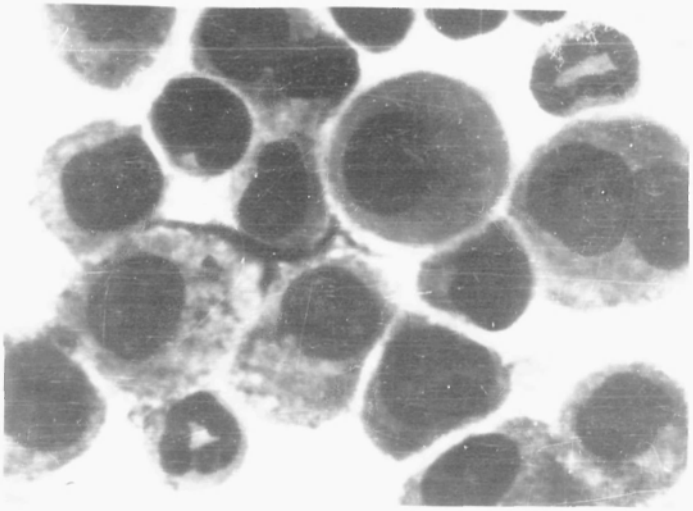
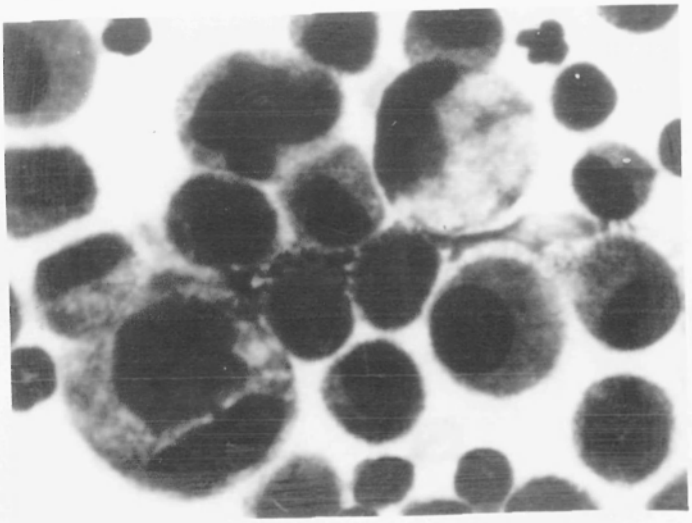


FIG.15.



to decline and all the rats acquired the latent state. It is apparent, therefore, that spleen is not responsible for the removal of these circulating microfilariae.

c. The course of infection after splenectomy

In this experiment 16-20 adult worms were transplanted into the thoracic cavity of 3 rats which were splenectomized 6 days ago. The unsplenectomized rats similarly transplanted served as controls. Microfilariae counts were made on these animals 7 days after the transplant and thereafter once a week till the disappearance of microfilariae from the peripheral blood.

The results show (Table XV) that in the splenectomized rats the infection ran a similar course as observed in intact controls.

TABLE XV

Effect of splenectomy on the microfilarial level in the peripheral blood of 3 rats, in which worms were transplanted in the thoracic cavity.

| Rats | Weeks after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | | |
| | 1 | 3 | 6 | 8 | 10 | 12 | 14 |
| Splenectomized | 87 | 220 | 1329 | 490 | 0 | 0 | 0 |
| | 166 | 400 | 810 | 260 | 60 | 0 | 0 |
| | 44 | 178 | 783 | 440 | 109 | 52 | 0 |
| Normal | 143 | 360 | 880 | 616 | 114 | 0 | 0 |
| | 97 | 322 | 535 | 276 | 0 | 0 | 0 |
| | 86 | 336 | 1119 | 705 | 229 | 0 | 0 |

11. Effect of partial pneumonectomy

Five rats with latent infection, 3 weeks after the termination of the initial infection, were partially pneumonectomized (right lung was completely removed) in order to see whether or not such rats became positive. Slides were taken 3 days after the operation and thereafter twice a week. It was found that partial pneumonectomy did not result in the suppression of acquired immunity as no microfilariae appeared in the peripheral blood of these rats for a period of 1 month when the observations were discontinued.

G. Immunosuppressive agents in the breakdown of acquired resistance

1. Cortisone

a. The effect of cortisone treatment on the density of microfilariae in rats with declining infection

A group of 8 infected rats, in which microfilaremia was declining, weighing 200-250 g each, was injected with cortisone acetate at a dose of 50 mg per kg per day for 13 days. Microfilariae counts were made 3 days after the start of the treatment daily for a period of 20 days (Table XVI).

TABLE XVI

Effect of injections of cortisone on the microfilarial level of the peripheral blood of eight rats with declining infection.

| Peak Microfilaremia | Microfilariae count before treatment. | Days after cortisone treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | Microfilariae per 10 cmm of blood | | | | | |
| | | 3 | 6 | 9 | 12 | 15 | 20 |
| 1607 | 34 | 57 | 190 | 294 | 198 | 352 | 1248 |
| 1030 | 2 | 1 | 1 | 3 | - | - | - |
| 930 | 20 | 15 | 18 | 80 | 145 | 180 | 250 |
| 1758 | 98 | 110 | 175 | 295 | 407 | 395 | 850 |
| 1000 | 51 | 41 | 60 | - | - | - | - |
| 580 | 15 | 15 | 16 | 22 | 19 | 14 | 20 |
| 886 | 3 | 2 | 3 | 7 | 9 | 6 | 2 |
| 764 | 12 | 10 | 21 | 25 | 37 | 30 | 21 |

- Counts could not be recorded further owing to the death of the animal.

By 15-20 days after the start of the experiment, 4 out of 6 untreated infected rats serving as controls, had developed resistance as shown by the total disappearance of microfilaremia, while in the remaining controls microfilaremia continued to decrease. In the treated rats, there was an increase in microfilaremia which continued at a high level until the termination of the experiment.

b. <u>The effect of cortisone treatment on rats with latent infection</u>

A group of 6 rats, with latent infection, weighing 200-280 g each, 1-2 weeks after the termination of the infection,

wrs injected with cortisone at the same concentration as before, daily for 13 days. Microfilariae counts were made 4 days after starting the treatment and thereafter at regular intervals. Two rats with latent infection were kept as untreated controls. Microfilariae appeared in the blood of treated rats one week after the start of treatment (Table XVII) while untreated controls had consistently negative counts.

TABLE XVII

Microfilarial level in the peripheral blood of 6 rats with latent infection after treatment with cortisone.

| Days after starting cortisone treatment | | | | | |
|---|---|---|---|---|---|
| Microfilariae per 10 cmm of blood | | | | | |
| 4 | 7 | 10 | 12 | 16 | 19 |
| 0 | 1 | 2 | 12 | 16 | 39 |
| 0 | 0 | 1 | 1 | - | - |
| 0 | 4 | 2 | 0 | 2 | 0 |
| 0 | 1 | 2 | 1 | - | - |
| 0 | 1 | 6 | 4 | 6 | 10 |
| 0 | 2 | 4 | 10 | 9 | 3 |

- Counts could not be recorded further owing to the death of the animal.

11. Whole-body irradiation

Experiments were designed to determine the effect of total-body irradiation on both the development and maintenance of acquired immunity. Accordingly, in the first experiment

a group of 7 rats was irradiated with 800 r, 24 hours prior to transplant into the thoracic cavity with adult worms. Non-irradiated rats similarly transplanted served as controls. Microfilariae counts were done 7 days after the transplant and thereafter regularly at weekly intervals.

Inspection of the data revealed that there was an interference in the development of acquired immunity as shown by the prolongation of infection in irradiated rats by 1-4 months as compared to controls.

Another experiment was designed to test the effect of irradiation on infected rats with microfilaremia in declining phase and the rats with latent infection. Eight infected rats and 6 rats with latent infection were irradiated with the same dose as given above. Microfilariae counts were made daily in case of rats with latent infection and twice a week for the rats with declining infection. Blood of rats was taken before and 7 days postirradiation and sera compared for the precipitin bands in the micro-gel diffusion plates. The results showed that irradiation does not affect the preformed antibodies as there was no significant difference in the number of bands before and after irradiation, but interfers with the development of acquired resistance to infection as would appear from the increase in microfilariae count of infected rats (Table XVIII). From the appearance of microfilariae (Table XIX) in the peripheral blood of rats

TABLE XVIII

Effect of irradiation on the microfilarial level in the peripheral blood of eight rats with declining infection

| Microfilariae count before irradiation | Days after irradiation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | | | |
| | 3 | 7 | 10 | 13 | 16 | 20 | 23 | 30 |
| 2 | 0 | 1 | 0 | 0 | 1 | 2 | - | - |
| 78 | 108 | 98 | 100 | 104 | 100 | 173 | 66 | 27 |
| 27 | 23 | 50 | - | - | - | - | - | - |
| 3 | 1 | 1 | 2 | 1 | 3 | 11 | 8 | 6 |
| 5 | 1 | 7 | 5 | 4 | 1 | 3 | 0 | 0 |
| 3 | 5 | 21 | 6 | 15 | 9 | 1 | 2 | 3 |
| 16 | 12 | 18 | 20 | 26 | 17 | 11 | 9 | 3 |
| 1 | 3 | 5 | 2 | 2 | 4 | 3 | 1 | 0 |

- Counts could not be recorded further owing to the death of the animal.

TABLE XIX

Effect of irradiation on the microfilarial level in the peripheral blood of five rats with latent infection.

| Days after irradiation | | | |
|---|---|---|---|
| Microfilariae per 10 cmm of blood | | | |
| 3 | 7 | 10 | 13 |
| 0 | 9 | - | - |
| 0 | 0 | 3 | 1 |
| 1 | 1 | 1 | - |
| 0 | 2 | 0 | 0 |
| 2 | 1 | 0 | 0 |

- Counts could not be recorded further owing to the death of the animal.

with latent infection, it can be seen that irradiation also adversely affects to significant extent the already developed acquired resistance.

iii. Antilymphocyte serum

Five rats with latent infection, 1-2 weeks after they developed latent infection as revealed by the absence of microfilariae in the peripheral blood, were injected with 1 ml of ALS into the thoracic cavity (0.5 ml in each cavity). Proper controls of rats with latent infection receiving same amount of normal rabbit serum were set up. Slides were made 5 hours after the injection and then daily for a period of 13 days. The rats were injected again with 0.5 ml of ALS on the third and fifth day starting from the day of treatment in a similar way. The results are presented in Table XX.

TABLE XX

Microfilarial level in the peripheral blood of five rats with latent infection after treatment with ALS.

| Days after ALS treatment | | | | | | |
|---|---|---|---|---|---|---|
| Microfilariae per 10 cmm of blood | | | | | | |
| 1 | 3 | 5 | 7 | 9 | 11 | 13 |
| 0 | 0 | 0 | 1 | 1 | 1 | 2 |
| 0 | 1 | 4 | 3 | 7 | - | - |
| 0 | 0 | 0 | 2 | 3 | 3 | 9 |
| 0 | 0 | 3 | - | - | - | - |
| 0 | 0 | 1 | 0 | 3 | 2 | 1 |

- Counts could not be recorded further owing to the death of the animal.

The data shows that microfilariae appear in the blood of rats, which had shown none prior to treatment, indicating thereby breakdown of the already developed acquired resistance. The rats injected with normal rabbit serum gave negative results.

The thoracic contents of the rats with latent infection were mixed with ALS to see its effect in vitro. It was observed that with the addition of ALS microfilariae were able to free themselves from the attached cells.

H. Possible sites involved in the disappearance of circulating microfilariae

In a group of 7 rats with latent infection with varying initial peak microfilaremia blood or pleural washings (1-2 ml) of infected rats with numerous microfilariae was injected intravenously or intracardially, after a smear had been made for microfilariae count to reveal the intensity of infection. In male rats weighing about 150 g or more successful injections were made into the femoral vein of the penis.

The results showed that in rats with latent infection intravenous injections of microfilariae resulted in a complete disappearance of the microfilariae in 2-20 days whereas in controls they circulated for a period of about 50-120 days.

In another experiment 10 normal rats weighing 150-250 g were injected with 1-2 ml of saline washings from the pleural cavities of rats with latent infection containing numerous microfilariae trapped with cells. The donor rats were chosen which had a high level of microfilaremia in the peripheral blood at the peak phase. Five uninfected rats were injected

with thoracic washings containing approximately same number of microfilariae from rats with patent microfilaremia. Slides were taken 5 minutes after the injections and thereafter every 30 minutes. It was found that microfilariae in the peripheral blood of rats, which had received pleural washings from rats with latent infection, promptly disappeared within 1-3 hours after the injection whereas in controls they circulated for a period of 60-120 days. These results strongly suggest another site which is responsible for the destruction of microfilariae. When the various tissues of the rats, which got the thoracic washings of the rats with latent infection, were examined soon after the disappearance of microfilariae, it was found that the microfilariae were concentrated in lungs and to some extent in liver. However, 4-8 hours after the injection no microfilariae were found in any of the tissues suggesting rapid destruction possibly in lungs and liver.

Thus, in rats that begin to acquire resistance, it appears, that in addition to the cellular infiltrate destroying the microfilariae, which are released in large numbers by female worms in the thoracic cavity, the circulating microfilariae are also trapped and cleared by lungs and liver.

I. Resistance to reinfection

Ten rats with latent infection (weight 180-260 g) of varying initial peak microfilaremia (1-3 weeks after the disappearance of microfilariae from the peripheral blood) and 10 normal rats were used for this study. All the rats were shaven with an electric clipper and exposed, overnight, to the mites containing infective larvae. The experimental and the control rats were kept together in the same desiccator containing mites, so as to have equal chances of bites by infective mites. Next day they were brushed off to remove adhered mites and kept separately in clean cages for further observation.

Blood smears were examined at weekly intervals, 40 days after exposure to infective mites, for a maximum period of 8 weeks, when the studies were discontinued.

The results reveal that rats in the latent state, infected second time showed a high degree of resistance when compared with adult controls infected at the same time (Table XXI). It can be seen that quite high level of microfilaremia appeared in the blood of adult controls showing that mature albino rats could be infected through mites. Moreover, microfilariae started appearing about 60 days after the infection, which is the normal incubation period, and persisted for several weeks. It will be seen from the

TABLE XXI

Microfilorial level in the peripheral blood of ten rats with latent infection and ten normal rats on mite-induced infection

| Rats | Weeks after the incubation period | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| With latent infection | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 7 | 14 | 10 | 5 | 2 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 1 | 8 | 16 | 17 | 14 | 3 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 8 | 32 | 6 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Normal | 0 | 2 | 278 | 196 | 276 | 641 | 760 | 816 |
| | 0 | 0 | 0 | 1 | 11 | 36 | 61 | 90 |
| | 0 | 0 | 17 | 139 | 390 | 1014 | 1150 | 1400 |
| | 0 | 2 | 25 | 69 | 170 | 290 | 340 | 289 |
| | 0 | 0 | 4 | 36 | 97 | 186 | 400 | 630 |
| | 0 | 0 | 0 | 2 | 23 | 66 | 83 | 100 |
| | 0 | 0 | 0 | 8 | 18 | 310 | 600 | 740 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 13 |
| | 0 | 1 | 18 | 27 | 40 | 109 | 132 | 170 |
| | 0 | 2 | 8 | 9 | 27 | 64 | 68 | 105 |

Table that out of 10 rats with latent infection, 3 showed a low level of microfilariae which disappeared altogether within few weeks.

In the above experiment resistance to reinfection was assessed purely on the appearance of microfilariae in the blood of the rats. All the rats were subsequently killed after 8 weeks of observation. At autopsy it was found that infective larvae in all the rats with latent infection developed to the adult stage. The worms, when kept at 37°C in normal saline for 15 minutes, could easily be separated by differences in size of the worms of initial infection. It was noted that young worms of the re-infection were present in the thorax of rats with latent infection and in numbers comparable to that seen in controls. It is apparent from this experiment that infective larvae, despite the humoral antibodies in high titer in a rat with latent infection, migrated to the thorax and developed there to the adult stage and produced microfilariae which could not penetrate the thorax, as they were not seen in the peripheral blood.

In the course of these experiments an interesting observation was made which deserves special mention. In 2 rats with latent infection about 30 infective larvae, which were removed from infected mites into Tyrode's solution,

were put directly into the thoracic cavity. When the rats were killed 2-3 days later, few infective larvae which were recovered, were found trapped with cells within the thorax. However, the larvae were very motile eventhough the cells were adhered to their body from both the sides. Whether this trapping of infective larvae, directly introduced into the thoracic cavity, leads to their destruction in a rat with latent infection was not determined in the present experiments.

J. Effect of protein malnutrition on the development of latent infection

The data on the effect of low and high protein diet on the development of acquired immunity is summarized in Table XXII. This was studied by maintaining the rats on protein deficient diet for 22 weeks. Obviously, the growth rate for the animals on the deficient diet was markedly lower than that of the other two groups.

The results show considerable interference in the development of acquired immunity in rats on low protein diet as revealed by prolongation of the period of microfilaremia by 4 weeks compared to rats on standard and high protein diet. Further, there is no significant difference in the time taken for the development of resistance in rats on high protein diet and in controls, assessed by the disappearance of microfilariae in both the groups 9-11 weeks after transplantation of worms.

TABLE XXII

Microfilarial level in the peripheral blood of seven rats on 6% protein diet, seven rats on 26% protein diet and seven rats on 15% protein diet, in which worms were transplanted in the thoracic cavity.

| Protein content of the diet % | Weeks after transplantation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | | | | | | | |
| | 1 | 3 | 4 | 5 | 7 | 9 | 10 | 11 | 13 | 15 |
| 6 | 85 | 338 | 566 | 701 | 360 | 317 | 269 | 120 | 0 | 0 |
| | 58 | 388 | 459 | 637 | 348 | 109 | 115 | 89 | 41 | 0 |
| | 88 | 483 | 623 | 567 | 755 | 697 | 763 | 673 | 436 | 70 |
| | 95 | 136 | 131 | 224 | 473 | 320 | 455 | 410 | 115 | 35 |
| | 33 | 183 | 151 | 428 | 370 | 290 | 115 | 84 | 30 | 0 |
| | 178 | 1447 | - | - | - | - | - | - | - | - |
| | 150 | 965 | 1230 | - | - | - | - | - | - | - |
| 15 | 11 | 35 | 18 | 13 | 7 | 1 | 0 | 0 | 0 | 0 |
| | 30 | 217 | 187 | 204 | 34 | 9 | 4 | 0 | 0 | 0 |
| | 60 | 806 | 387 | 313 | 63 | 7 | 0 | 0 | 0 | 0 |
| | 7 | 107 | 184 | 150 | 134 | 34 | 5 | 0 | 0 | 0 |
| | 6 | 104 | 91 | 156 | 146 | 13 | 2 | 0 | 0 | 0 |
| | 138 | 1038 | 616 | 332 | 61 | 0 | 0 | 0 | 0 | 0 |
| | 26 | 250 | 349 | 535 | 300 | 157 | 47 | 0 | 0 | 0 |
| 26 | 14 | 63 | 55 | 42 | 3 | 2 | 1 | 0 | 0 | 0 |
| | 7 | 44 | 29 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 76 | 523 | 321 | 281 | 29 | 9 | 1 | 0 | 0 | 0 |
| | 2 | 10 | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 86 | 43 | 52 | 15 | 43 | 6 | 0 | 0 | 0 |
| | 43 | 600 | 175 | 139 | 78 | 19 | 14 | 0 | 0 | 0 |
| | 74 | 1027 | 840 | 633 | 355 | 28 | 0 | 0 | 0 | 0 |

- Counts could not be recorded further owing to the death of the animal.

Three rats on low protein diet were challenged with adult worms after the development of latency. It was found (Table XXIII) that such rats do not show any microfilariae eventhough maintained on the deficient diet throughout the experiment, which suggest that in these rats the initial infection was prolonged but they do develop a high degree of resistance to further challenge at a later stage.

TABLE XXIII

Microfilariae in the peripheral blood of three rats that developed latent infection after maintenance on low protein diet (from previous experiment, Table XXII) and three normal rats, on transplantation with worms in the thoracic cavity.

| Rats | Days after transplantation | | | |
|---|---|---|---|---|
| | Microfilariae per 10 cmm of blood | | | |
| | 1 | 4 | 7 | 10 |
| With latent infection | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| Normal | 6 | 19 | 66 | 105 |
| | 2 | 32 | 110 | 319 |
| | 1 | 7 | 28 | 53 |

K. Neonatal infection and development of acquired resistance.

Sixteen newborn rats were exposed to infected mites on the day of birth. The method of infecting the newborn was to attach about 5-10 mites picked up randomly from an infected desiccator, while the rats were kept separately in beakers. A few mites before attaching were dissected to find out the average rate of infectivity, which was found to be 6 larvae per mite. The mites, when fully fed, were removed from the baby rats after about 2 hours. After the incubation period of 40 days slides were taken and examined for microfilariae and thereafter every week for a period of 20 weeks alongwith the blood from 20-day old rats infected at the same time and under similar conditions for comparison. It was observed that the microfilariae started appearing in rats infected in early life approximately at the same time as in controls. The observations indicated that there was no tolerance in neonatally infected rats to microfilariae as in most of the rats microfilaremia started declining 18 weeks after the infection and all the rats developed latent infection in about 6 months after the infection (Table XXIV). This was the normal period taken by the controls to clear all the microfilariae from the peripheral blood. When the microfilarial level in the peripheral blood dropped to zero all the rats were killed and autopsied. A marked cellular infiltration was noted and the microfilariae were found to

TABLE XXIV

Microfilarial level in the peripheral blood of sixteen rats which were infected through mites at the neonatal stage.

| Weeks after the incubation period | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Microfilariae per 10 cmm of blood | | | | | | | | | |
| 2 | 3 | 6 | 8 | 10 | 11 | 13 | 16 | 19 | 20 |
| 0 | 18 | 60 | 233 | 29 | 0 | 0 | 0 | 0 | 0 |
| 0 | 35 | 330 | 564 | 612 | 1000 | 650 | 210 | 18 | 0 |
| 0 | 85 | 240 | 810 | 1240 | 1030 | 714 | 410 | 116 | 0 |
| 0 | 10 | 29 | 70 | 160 | 93 | 35 | 2 | 0 | 0 |
| 0 | 0 | 2 | 21 | 18 | 14 | 9 | 1 | 0 | 0 |
| 0 | 17 | 169 | 430 | 1200 | 1020 | 650 | 336 | 70 | 2 |
| 0 | 6 | 30 | 209 | 270 | 666 | 286 | 10 | 0 | 0 |
| 0 | 3 | 10 | 28 | 30 | 5 | 0 | 0 | 0 | 0 |
| 0 | 26 | 140 | 677 | 1183 | 1537 | 792 | 528 | 30 | 7 |
| 0 | 5 | 29 | 260 | 232 | 166 | 36 | 14 | 0 | 0 |
| 0 | 2 | 8 | 42 | 66 | 40 | 7 | 1 | 0 | 0 |
| 0 | 8 | 102 | 1168 | - | - | - | - | - | - |
| 0 | 21 | 62 | 279 | 565 | 380 | 207 | 90 | 0 | 0 |
| 0 | 0 | 3 | 4 | 8 | 3 | 0 | 0 | 0 | 0 |
| 0 | 2 | 130 | 472 | 921 | 561 | 450 | 78 | 0 | 0 |
| 0 | 12 | 186 | 730 | 1190 | - | - | - | - | - |

- Counts could not be recorded further owing to the death of the animal.

be trapped with cells and undergoing destruction in the thorax in the neonatally infected rats in the same manner as seen in controls. It is, therefore, concluded that neonatal infection of L. carinii in rats does not lead to immunological unresponsiveness.

L. Susceptibility of mice to L. carinii infection

Twenty 1 month old albino mice were exposed to infective mites. Seven days after the infection 3 mice were killed to see whether the infective larvae develop or not. It was found that the larvae were in the process of development as 15-20 immature worms were seen in the thorax of each mouse. Their lengths were from 1-1.6 cms. Forty days after infection the blood was examined for microfilariae and thereafter every fortnight for a period of 9 weeks. It was found that all the mice did not show any microfilariae in the peripheral blood. Ten weeks after the infection 5 mice were killed but only dead and calcified worms were found in the thorax. At autopsy 15 weeks after the infection when the experiment was terminated, encapsulated mass containing parts of adult worms was found in the thorax of a few mice indicating that the larvae migrated succesrfully to the thorax, developed there to a certain stage, but were later calcified and fragmented. In most of the cases nothing was seen in the thorax as well as in the peritoneal cavity.

In another experiment albino mice were transplanted with adult worms, obtained from infected rats, into the thoracic cavity. It was found that all the mice transplanted directly with adult worms developed microfilaremia in the peripheral blood 1 day after the worm transplant and the microfilariae remained in the blood for 3 months. The adult worms, however, in all the mice were found to be dead by this time. A few mice were again transplanted to see if the acquired resistance had developed that might be responsible for the disappearance of microfilariae. The results, however, showed that mice became positive and developed microfilaremia as in control, uninfected mice, transplanted with the same number of worms. It shows that microfilarial disappearance in mice was not a result of acquired resistance. Humoral antibodies to adult worms were detected in these animals in gel diffusion plates.

## DISCUSSION

A battery of immunologic tests conducted in the past in humans in endemic areas by several workers with antigen extracts from filarial parasites emphasized their nonspecificity and considerable cross reactivity. Methods for specific diagnostic purpose require a relatively pure antigen like that used by Sawada et al. (1962) rather than the complex antigens used by previous investigators (Kagan, 1963a; Schiller, 1967). Sawada and Takei (1965) were among the first to study the immunologic reactions with purified D. immitis antigen in human filarial cases. Although the nonspecific reactions were found to be reduced on fractinating the antigens, nevertheless, they still persist to a certain extent. Tada and Kawashima (1964) used a purified antigen from D. immitis and found no correlation between microfilarial density and wheal size in microfilarial carriers. Cross reactions were found in 1 in 15 individuals with gnathostomiasis and 1 in 17 with schistosomiasis but not in patients with other common helminthic infections. However, if a group specific antigen is used in testing humans, false positive reactions due to zoonotic filariasis must be kept in view. Augustine and Lherisson (1946) observed that the positive skin reactions with D. immitis antigen with individuals without any evidence of filarial infection are due to non-human filarial worms which

sensitized man. Garcia *et al.* (1968) also reported, using antigen obtained from the adults and microfilariae of *D. immitis*, false positive reactions in individuals presumed not to have been exposed to filariasis risk. The data of the present study further emphasize the importance of extensive fractionation of antigens, removal of unwanted common antigens and use of purified antigen(s) in evaluating their serodiagnostic value.

The results of the present investigation, in general, demonstrate the antigenic complexity of *L. carinii* and other species examined. The number of antibodies observed in infected rat serum was less than that in serum from artificially immunized rabbit as the latter were exposed to a greater number of antigens of the parasite. Augustin *et al.* (1963) demonstrated at least 7 antigens in extracts of *L. carinii* in immunoelectrophoresis. They compared antibody response in cotton and Delhi rats, using gel diffusion and passive haemagglutination tests, and found that although cotton rats were more heavily infected than Delhi rats, less antibodies were produced by cotton rats than Delhi rats. Cross reaction studies between *L. carinii*, *D. repens*, *D. immitis* and *Ascaris* in the present investigation suggest the presence of sensitizing antigens common to all. The present data further indicate that while several antigen

fractions were common among the various species, species specific fractions were also present in L. carinii and other parasite extracts.

In order to evolve at a reliable serodiagnostic test for filariasis, an ideal animal parasite of choice must be the one which shares an antigen or antigens only with human parasite and not with other species or related helminths. Further, to eliminate false positive reactions it may be more rewarding to look for a common antigen between L. carinii and human parasite because mite being the vector for L. carinii the chances of infected mite, unlike infected mosquito, sensitizing normal healthy individuals are remote. Hence with the purified antigen from rat parasite one may not expect false positive and cross reactions in healthy individuals even in an endemic area. It is encourging in this connection to note the work of Culbertson et al. (1947), Oliver-Gonzalez (1953) and Ishii and Tanaka (1968) which suggest the presence of group specific antigens between L. carinii and human parasite W. bancrofti. Ishii and Tanaka (1968) reported that L. carinii antigen cross-reacted with filariasis suspected human sera up to 64 dilution in indirect fluorescent antibody technique and upto 2,000 dilution using indirect haemagglutination test.

PCA test is the most sensitive *in vivo* method for determining antigen-antibody reactions. Although it is a relatively simple technique, its value has not been seriously evaluated in parasitic diseases. The evidence shown here of PCA reactions being positive even at a very high dilution further emphasizes the value of this method for detecting low antibody concentration. Recent studies have shown that PCA is mediated by two different kinds of antibodies. In one, that is known as heterologous or heterocytotropic anaphylaxis, the anaphylactic antibody from one species produce PCA reactions in the skin of another species, e.g. rabbit serum in guinea pig skin. This type of PCA reaction is positive within 4 hours after the passive sensitization of the skin, but is negative at a latent period of 48 hours, and is mediated by $7S\gamma_2$ globulin. This is the type of antibody described by Ivey & Slanga (1965) in rabbits infected with *Trichinella* or *Toxocara*. The rabbit antibody to *L. carinii* antigen belong to this class of antibodies. The second kind is called homologous anaphylaxis or homocytotropic anaphylaxis in which the antibody gives PCA reactions in the skin of the species from which the antibody is derived e.g. rat antibody in rat skin. It is mediated by antibodies which appear to be due to $7S\gamma_1$ immunoglobulins. The test is positive when the antigen is injected 4 hours after the passive sensitization. Another homocytotropic antibody which is gaining increasing

importance in helminthic infections is the reaginic type of antibody which gives PCA on injection of antigen after a latent period of 48-72 hours, but the antibody remains fixed to the skin for many days or weeks (Zweifler & Becker, 1966). The results show that infected rats are capable of producing anaphylactic antibodies which give PCA only in rats and not in guinea pigs. Further, it was found that there are two different types of homocytotropic antibodies formed as in some rats the antibody could be detected at 4 hours after sensitization and in others at 72 hours, whereas in some both the types were present. Mota (1963a) reported that in the rat, homologous PCA can be induced both by 'reagins' and by an antibody found in serum with high levels of precipitating antibody. The antibodies found in the precipitating serum give PCA at 6 hours, but not at 72 hours. It has recently been found that a new class of human immunoglobulin, IgE has the ability to induce or block homologous passive skin sensitization (Stanworth et al., 1967; Bennich et al., 1968). It thus appears that IgE is a carrier of reaginic antibody in humans. One of the skin sensitizing antibody of rat to L. carinii belongs to the antibody recently described by Ogilvie (1967) in rats infected with N. brasiliensis which gives PCA at 72 hours. Similar reports are found in the literature for the presence of reagins in other helminthic infections such as

T. colubriformis (Ogilvie, 1964), S. mansoni (Ogilvie et al., 1966), D. uniformis (Sadun et al., 1967), T. spiralis (Sadun et al., 1968), and O. ostertagi in sheep (Hogarth-Scott, 1969). It is believed that, in general, helminths stimulate the host in the course of infection or by environmental contact to produce reagins (Hogarth-Scott, 1967a). These findings have been recorded not only in man but also in the rat (Ogilvie, 1964; Wilson, 1967), rabbit (Hogarth-Scott, 1967b), dog (Rockey & Schwartzman, 1967), monkey (Ogilvie et al., 1966; Ishizaka & Ishizaka, 1968), guinea pig and mouse (Bloch, 1967), and sheep (Hogarth-Scott, 1969).

The presence of two antibodies in infected rat serum which are not present in immunized rabbit serum can not be explained at present. These may arise due to certain excretory antigenic products of the parasites or to antigens present in a different developmental stage of the parasite which exist in minor amounts in the adult stage and were not able to elicit detectable antibody response in rabbits.

The anti-adult precipitins were first detected on the 7th day after infection in infected rats, which increase gradually and persist throughout the course of infection. The early appearance of antibodies in this infection seems to differ from the antibody response of dogs infected with D. immitis. Pacheco (1966) followed antibody response of dogs infected with D. immitis at short intervals using a

complement-fixation test and showed that antibodies first appeared in the circulation when immature worms start migrating to the heart during the third month of infection. However, antibody titers were noted as early as 3 weeks after infection using indirect haemagglutination test. Antibody titers then increased rapidly, remained at a peak, and decreased gradually with the appearance of microfilariae in the blood and were hardly detectable by the 9th month of infection.

Previous workers have found albino mice unsuitable as an alternative host for L. carinii infection (Hawking & Burroughs, 1946; Wenk and Heimburger, 1967). Sen and Bhattacharya (1961) reported that black mice are refractory to infection with L. carinii. The results of the present study are in accord with the conclusions of the above workers. The observations indicated that albino mice exposed to natural infection of L. carinii through mites did not show any microfilariae in the peripheral blood as the adult worms, if found, were calcified. At what stage the worms were fragmented was not determined in the present study. Wenk and Heimburger (1967) reported similar results of finding adult worms in the pleural cavity of albino mice infected by bites of the vector, but no microfilariae appeared in the peripheral blood. In mice infected in the neonatal stage encapsulation of adult worms occurred late or only partially. Similar results were noted by transplantation of metacyclic

larvae in the thoracic or abdominal cavity of adult mice. Further, it was reported that transplantation of adult worms in adult mice gave a temporary microfilaremia in the peripheral blood. The results of the present study show that microfilariae appear in large numbers and persist for at least 3 months in the peripheral blood of mice transplanted into the thoracic cavity with adult worms. These findings are in contrast to the results obtained by Wenk and Heimburger (1967) where only temporary microfilaremia was observed on transplantation with adult worms. However, it may be due to the susceptibility of different strains of mice as it is well known that the susceptibility of different strains of the same species is different to one and the same infection. Further, the observations of the present study would indicate that although mice could be infected by direct transplantation of adult worms and microfilaremia produced, the adult worms appear to live a relatively short time in this unnatural host. It appears the production of microfilariae is continued by the worms undergoing calcification to a certain period before they are completely fragmented.

The observations in mice transplanted after the disappearance of microfilariae of an initial worm transplant indicated that mice do not develop acquired resistance to a further challenge. However, circulating antibodies were formed in response to the infection, that seem to be

ineffective in killing the microfilariae as they continued to increase in the peripheral blood of the host after a second worm transplant despite the presence of preformed antibodies in the blood in high amounts. It may be concluded that the microfilariae do not stimulate acquired resistance in the mice and will merely live out their span of life and the death of the adult worms is probably the reason for the fall in microfilaremia and the total disappearance after a certain period.

The level of irradiation determines the degree of deleterious changes seen in the infective larvae and their subsequent behaviour in the host. The present data shows that irradiation upto a level of 80,000 r did not affect the immediate survival of larvae or the mite to any appreciable extent.

Gould et al. (1953) found that in vitro a dose of 750,000 r killed the larvae of T. spiralis, whereas a dose of 5000 r - 6,000 r inhibited development of larvae to the adult stage, and a dose of 3,500 r sterilized the worms. It is believed that, in general, an increasing percentage of infective larvae are damaged with increase in radiation dose and the minimum radiation level required for complete or near complete destruction, varies from species to species. Although irradiation upto 80,000 r had no appreciable effect on the infective larvae of L. carinii, their behaviour and

fate in the host was affected even at much lower levels of irradiation. It appears that larvae irradiated at 20,000, 40,000, 60,000 and 80,000 r lose their ability to develop into adult worms in the host and it seems that they are ultimately destroyed as no trace of worms was found in the thorax when searched 16 days after infection. The fate of irradiated larvae, how exactly they were destroyed, was not determined in the present experiment. The rats did not develop resistance against challenge with normal infective larvae although humoral antibodies were detected after infecting the rats with irradiated larvae which suggests the successful penetration and migration of these larvae in the host. It is possible that it is not the young infective larvae which give rise to the immunogenic stimulus.
The following possibilities could be given to account for the failure in stimulating resistance with irradiated larval vaccine of L. corinii in the present study: (i) that the larvae had been over irradiated and had failed to mature to the immunizing stage, (ii) the number of infective larvae entering into the host was small and (iii) booster doses with irradiated larvae may be important in producing a

degree of immunity sufficient to stand up a challenge. Probably, it is worthwhile to investigate with doses smaller than 20,000 r whether it induce immunity against challenge with normal infective larvae.

On the basis of the findings of the present study, there is now sufficient information to throw light on several problems associated with the nature of the stimulus and the factors involved in the development of acquired immunity to L. carinii in the albino rat. The results show that the infective larvae and the adult worms and/or their metabolic products are not responsible for the major stimulus to resistance in this host-parasite system. Rats do not develop immunity until the infection has reached maturity and microfilariae pass through thorax. The interval of about 5-6 months between infection and the development of immunity coincide with the production of many microfilariae by female worms. Worms killed with dry ice immediately before transplant did not stimulate resistance although freezing causes minimal denaturation of the protein antigens. Further, the resistance cannot be induced by transfer of male worms. All rats transplanted with 10-15 female worms developed immunity to further transplant of a comparable size while the rats transplanted with as much as 100 or more males showed microfilaremia when transplanted with female worms as shown in Table VII. The lack of immunity,

by transplantation of female worms exhausted of microfilariae by serial passage in rats for a short time, alongwith the results discussed above further indicate that microfilariae provide the major stimulus to resistance. Direct evidence that the antigenic stimulus necessary for inducing resistance comes from the microfilariae was apparent when repeated injections of microfilariae into uninfected rats led to development of acquired resistance. These views are similar to those of Wong (1964b) that living microfilariae provide antigenic stimulus in Dirofilaria immunity.

The degree of immunity or resistance to infection depends on the size of primary worm burden and is roughly proportional to the intensity of infection which is indicated by peak microfilaremia. Although the microfilariae count may be somewhat unreliable to reveal the worm load present, since fecundity is a direct expression of successful establishment, normal development and continued life of the female worms, it would seem that the microfilariae count may be taken as roughly associated with the size of the initial infection.

The establishment of the adult worm in mature rats with latent infection after reinfection raises an interesting problem. Whatever the nature of the immune response it does not necessarily destroy established adult worms, or prevent them from producing microfilariae. In cotton rats infected

with L. carinii, it has been demonstrated that previous infection results in a degree of immunity which retards the growth and development of a challenge infection as compared to controls (Macdonald & Scott, 1953). The injection of dead larvae did not produce such an effect while fresh or dead adult worms did so. In the present experiments infective larvae introduced through mite-induced infection appear to develop to the adult stage in rats with latent infection. However, infective larvae introduced directly into the thoracic cavity of rats with latent infection, when examined, although active were found to be trapped with cells in the thorax. Whether there is significant growth in infective larvae before they reach thorax which protects them from the destruction by cellular infiltrate has not been ascertained.

It is interesting to find in this investigation that 6-7 months old albino rats were susceptible to mite-induced infection (Table XXI). This observation is in contrast to the results published by many workers in attempting to infect mature albino rats with L. carinii (Bertram, 1966, for review). Olson (1960a) found that 1-year old white rats were highly resistant for

*L. carinii* infection by subcutaneous introduction of infective larvae. Similar observations were made by Briggs (1963) who could not succeed in infecting mature white rats even by treatment with cortisone. This inability may be due to the difference in strains of white rats in their susceptibility to *L. carinii* infection as Dalip Singh and Raghavan (1962) were successful in infecting adult rats with *L. carinii*.

In order to explain the mechanism for the absence of microfilaremia in filarial infections, many workers in the past have advanced a number of possible explanations such as death of adult worms, sterilization of the worms due to some unknown factors including acquired immunity (Jordan, 1956), or prevention of microfilariae from reaching the peripheral blood or destruction after reaching it as a result of humoral antibodies (Kershaw, 1949b). Manson (1899) suggested that elephantiasis causes a blockage of microfilariae unless infection is extensive or reinfection common. Napier (1944) reported that microfilariae retained in the lymphatic vessels or in the subcutaneous tissues are actively destroyed and this provides the necessary sensitizing stimulus. Iyengar (1938) expressed similar ideas and suggested that the reaction may be based on the adhesion phenomenon of Pandit *et al.* (1929). Wilson (1948) reported that all the worms acquired after the onset of

elephantiasis are trapped in the obstructed lymphatics. Further, he suggests if there is a defensive mechanism at work, which either prevents the infective larvae from growing to adults, or keeps the adults sterile, it must have some connection with the attacks of lymphangitis. Wong (1964b) studied the immunologic response of uninfected dogs to injections of living microfilariae and inferred from his results that a relationship exists between the microfilaremia and the antibodies produced by the host in response to the presence of microfilariae, and that a high titer of such antibodies may be responsible for the absence of microfilariae in some filarial diseases.

In albino rats infected with *L. carinii*, Ramakrishnan *et al.* (1962) have shown that the development of latency was not due to death of adult worms, as these were found alive in the rats with latent infection, and further, these living worms were capable of producing microfilariae in uninfected rats. These observations were confirmed (Table XI) and extended by the present investigation.

Circulating antibodies can be detected by micro-gel diffusion technique in the sera of rats 7 days after infection which increase as the infection progresses. However, the protective nature of the antibodies could not be demonstrated. A criterion for determining the protective nature of antibodies is by observing the effect of immune serum on parasites and

in a number of cases the formation of precipitates at the body openings of the worms has been observed (Soulsby, 1962, for review). In N. muris infection in rats Sarles and Taliaferro (1936) showed that larvae administered to previously infected rats were trapped in the skin and lungs. Precipitates formed around the infective larvae of N. muris in vivo, similar to those found in vitro, when the larvae were placed in immune sera from previously infected rats. Wong (1964b) demonstrated by in vitro test, that immune sera taken from dogs on immunization with repeated injections of living microfilariae of D. immitis agglutinated homologous living microfilariae. Scott (1962) and Briggs (1958) noted precipitates around infective larvae of L. carinii when placed in sera of infected animals. McFadzean (1953) however, found no indication of the presence of agglutinins, precipitins or adhesins when microfilariae or infective larvae of L. carinii were incubated in immune sera in vitro and did not find any difference in the survival times of the larvae or microfilariae as compared to those incubated with normal sera. Further, studies of Scott (1960) suggest that immobilisation of the parasites by serum factors does not play a major role in the development of immunity. Similarly, Jackson (1959) confirmed the formation of precipitates by fluorescent antibody technique, which were

observed earlier by Oliver-Gonzalez (1940) studying the *in vitro* effects of immune serum on *T. spiralis*, but could find no evidence of parasiticidal activity against either larval or adult *T. spiralis*. Michel (1968) described that circulating antibody is not closely connected with immunity to helminths. The results of the present study also do not lend much importance to serum antibodies of rats with latent infection in the development of acquired immunity as no significant difference was found on the activity of microfilariae when incubated with hyperimmunized rabbit serum against whole *L. carinii* and sera from rats with latent infection compared to that incubated in sera from uninfected animals.

Although antibodies could be induced in uninfected animals by the injection of whole worm material in Freund's complete adjuvant these animals were not resistant to challenge infection either with direct transplantation of worms into the thoracic cavity or through natural infection by mites. McFadzean (1963) had a similar experience with dead *L. carinii* whole worm material. The injection of vaccines made from dead worm material has, in general, proved disappointing and it is now believed that functional antigens are associated with the living parasites which must live in the host to produce a satisfactory immune response (Terry, 1968).

The effect of circulating antibodies is ascertained, in general, by the ability of the antiserum in giving protection from infection on passive transfer. Several studies have been made of such a method of transferring immunity in a variety of helminth infections (Soulsby, 1962, for review). Stirewalt (1963) in a review on schistosome infections concluded that demonstration of protective schistosome antibodies has been elusive. She reported, like many others, unsuccessful attempts to passively immunize the recipients with high titer heterologous antisera. Ogilvie (1964) suggested that reaginic antibodies might be involved in immunity to helminths, as these antibodies appeared to be stimulated only by a living infection and passive transfer of immunity to S. mansoni could be achieved in rats by injecting small amounts of reaginic sera intradermally in the direct path of cercariae (Ogilvie et al., 1966). Hsu and Hsu (1966) reported that reagins are not involved in acquired resistance to S. japonicum. Recent attempts of passive immunity to T. spiralis in mice after using large volumes of serum with high antibody titers, and even whole blood were unsuccessful (Larsh, 1967a). Wong (1964b) observed that sera from dogs immunized with living microfilariae of D. immitis agglutinated homologous

microfilariae and prevented the production of microfilariae by adult worms in culture. She also observed reduction of microfilariae from the peripheral blood of infected dogs receiving injections of sera from such immunized animals. However, McFadzean (1953) failed to demonstrate passive transfer of immunity when rabbit antiserum prepared against whole L. carinii worm was given to cotton rats infected with L. carinii having large number of microfilariae in the peripheral blood. In the present experiments, as well, repeated injections of hyperimmune rabbit serum to L. carinii antigens and sera from rats with latent infection failed to reduce the microfilarial infection in rats, thus supporting the work of McFadzean that circulating antibodies may not play a significant role in filarial infection in rats. The precipitins, agglutinins, anaphylactic type of antibodies and reagins were present in the immune sera of animals with latent infection as demonstrated by various immunochemical techniques. The total quantity of the immune serum transferred in the present study in a rat of about 150-200 g weight was 7 ml and should be apparent if it conferred any degree of resistance to infection with L. carinii and if the circulating antibodies are protective in nature.

The data of the present study shows that splenectomy of rats before infection, at the declining infection or in

the latent state had no effect either on the development or in the breakdown of already acquired resistance. Duke (1960a), however, reported that splenectomy resulted in a rapid increase of microfilariae count in a drill infected with Loa loa which had suppressed infection before splenectomy. Splenectomy prior to infection resulted in a prolongation of microfilaremia that rose more or less steadily for about 12 months whereas it was suppressed after 8-12 weeks in intact controls. Hawking (1962) studied the effect of splenectomy on the microfilariae level in the blood of D. immitis and D. repens in dogs, L. carinii in cotton rats and D. witei in jirds. He reported that for L. carinii infection removal of spleen often resulted in a small increase in the number of microfilariae appearing in the circulation. However, on histological examination he could not find any evidence of microfilariae being destroyed in spleen and concluded that the marked destruction of microfilariae in the spleen of monkeys with loaisis was probably an exceptional phenomenon and that in other filarial infections spleen does not play a major role in destroying microfilariae. Wong (1964a) showed that splenectomy had no effect on the levels of microfilaremia in dogs infected with D. immitis. The results of the present study are in agreement with that of Ahmed (1967) who recently studied the role of spleen in destroying microfilariae of L. carinii in Liverpool white

rats, of sub-periodic B. malayi in golden hamsters, and of B. pahangi in cotton rats, Delhi white rats, and multimammate rats, but could not find histological changes in the spleen described by Duke (1960b) in loaisis and concluded that the spleen played no part in the destruction of microfilariae of L. carinii, sub-periodic B. malayi and B. pahangi.

Both the lack of demonstrable humoral antibody related to resistance, and the ineffectiveness of passive transfer suggested that trapping of microfilariae may be purely a cellular involvement and the antibodies responsible for acquired resistance may be cell-mediated. Further experiments provided sufficient evidence that cellular factors play an important role in this immunity. The experiment on cortisone treatment has shown that the drug breaks the acquired resistance which was manifested by appearance of microfilariae in rats with latent infection and an enhanced susceptibility revealed by an increased microfilariae count of rats treated with the hormone in a declining phase of the initial infection. The present studies show that this hormone administered daily over a specified period interfers with immune response to this parasite.

The mechanism of action of cortisone drugs is not known or incompletely understood at present, though the administration of these hormones is usually associated with

evidence of diminished local inflammatory response (Kass & Finland, 1953). Thomas (1953) suggested that the underlying mechanism is through blockade of the reticuloendothelial system thus preventing cellular defences coming into play. Wells (1962) suggests it impairs most cell functions. The suppression of the resistance already acquired is more difficult to understand. Weinstein (1955) and Coker (1956c) suggested that the main action of cortisone in resistance to helminths is on a cellular rather than on a humoral mechanism. Markell and Kerrest (1955) obtained evidence for inflammatory blockage of the lymphatic vessels in patients with elephantiasis, which prevented passage of microfilariae to the blood stream. These authors could reverse the condition to some extent by cortisone administration. Jordan (1960) reported a similar appearance of microfilaremia in a patient with elephantiasis and no evidence of microfilariae in the peripheral blood when treated with prednisolone for 3 weeks. It was suggested that corticosteroid may be involved in the reduction of acquired immunity responsible for the destruction of microfilariae. In the present experiments the action of cortisone in breaking the resistance of rats with latent infection having high titers of preformed antibodies shows that it is unlikely that the main action was on circulating antibody. It is evident from the findings of Fischel et al. (1951) that in rabbits the rate of metabolism of antibody is not affected by cortisone. It may be that the

direct effect of antibodies against microfilariae are ineffective by themselves in destroying the microfilariae. On the basis of the apparent interference with the acquired immunity it appears that cortisone probably exerts its effects by preventing the cellular infiltration into the thorax.

Irradiation provides a valuable tool to study the factors that influence the immunity of the host and ultimately to shed light on the mechanism involved in the operation of acquired immunity. Rats with latent infection exposed to whole-body irradiation of 600 r show interference with the maintenance of acquired immunity. The data indicated a successful penetration of some microfilariae as revealed by the appearance of a low level microfilaremia in rats with latent infection after irradiation, indicating thereby a partial breakdown of the immune response (Table XIX). However, in the rats irradiated before transplantation with adults worms, or infected rats having microfilariae in the declining phase, there was interference in the development of immunity, as measured by the prolongation of the microfilaremia for 1-4 months compared to controls. The time period between irradiation and infection, therefore, seems to be an important factor in determining to which extent interference occurs.

The factors involved in the depression of the acquired immunity by whole-body irradiation are not well understood. Medawar (1963) found that irradiation favours transplantation tolerance in adult mice. In the present experiments irradiation injury to lymphocytes may have accounted for the partial breakdown of the immune response in resistant rats and for the interference in the development of acquired immunity in actively infected rats and rats which had received irradiation prior to infection. This is to be expected in view of the reported sensitivity of lymphocytes to irradiation (Bloom, 1948). In T. spiralis infection in mice there is severe leucopenia due mainly to lymphopenia, which is noted 1-day postirradiation and continues for about 21 days before recovery (Yarinsky, 1961, 1962). Based on the serological responses and histopathological findings, Lerch et al. (1962) advanced a hypothesis to explain the mechanism of the irradiation interference in immunity to T. spiralis in mice. They concluded that the irradiation effect appears to be caused by damage to the haematopoietic system which is then unable to supply the large number of cells needed to initiate and/or maintain an effective inflammatory response in the area of worm elimination. Without an effective inflammatory response worms remain despite unaltered preformed antibody titers.

Antilymphocyte serum has assumed prominence as immunosuppressive agent for the prevention of homograft rejection (Woodruff & Anderson, 1964; Wolstenholme & O'Connor, 1967), and heterografts (Lance & Medawar, 1968) in experimental animals. Further evidence that cell-mediated immunity is responsible for the destruction of microfilariae was provided when ALS was found to free the trapped microfilariae from the attached cells *in vitro* and when injected into the thoracic cavity of rats with latent infection resulted in the appearance of microfilariae in the peripheral blood. Apparently, then there is a considerable suppression and slowing of the process by ALS treatment. Similar suppression of immune response by ALS has been recently reported by Kassai *et al.* (1967) in rats infected with *Nippostrongylus*. The formation of granuloma around *S. mansoni* eggs, which is due to delayed hypersensitivity (Warren *et al.*, 1967), could be completely suppressed by ALS (Domingo & Warren, 1968).

It is not clear how ALS causes immunosuppression. ALS contains antibodies of many specificities. A possibility is that cytotoxic antibodies specific for lymphocytes may result in causing immunosuppression or this suppression might be due to its combined effect on both the lymphocytes and macrophages.

The foregoing observations show that there is a definite relationship in rats between the cellular response and resistance to infection with L. carinii. Apparently, infection influences the host in some way so that there is a cellular infiltration which surrounds the microfilariae in the area of penetration. The activity of lymphocytes and macrophages may be the chief mechanism of resistance. Their defensive role seems therefore is to kill the microfilariae effectively. It is becoming increasingly evident that lymphocytes are potentially phagocytic cells (Bergman & Pollock, 1967; Hughes, 1966; Howard, et al., 1966; Holub, 1967; Metcalf, 1967) and may serve as a mobile source of macrophages to supplement local mechanism of defence (Bloom, 1928; Kolouch, 1939; Downey, 1966; Sieracki & Rebuck, 1960). The acquired resistance may be assumed, therefore, to depend upon the interaction between the lymphoid cells and the microfilarial antigens with which they are reactive. The fact that macrophages were found to adhere to the microfilariae suggests that these are also involved in addition to the lymphocytes in bringing about the acquired resistance to the infection.

It is believed that circulating microfilariae in the blood are harmless although they are often present in large numbers. It seems from the results of the present

investigation that intimate contact between host tissue and microfilariae affects particularly the microfilariae in the thorax which are first trapped and killed and thus provide necessary sensitizing stimulus for the destruction of microfilariae which are circulating in the blood at some other site possibly in the lungs and liver. The immunity has no effect on the development of the infective larvae of a second infection as there was no evidence that fewer worms developed as a result of reinfection. The trapping of the microfilariae in the thorax might be the sensitizing stimulus resulting in the development of acquired immunity. Studies of Chowdhury and Schiller (1962) with fluorescent antibody suggest that dead or damaged larvae are more immunogenic than live ones. In 'occult filariasis', which is a term used to designate a filarial infection in which no microfilariae are found in the peripheral blood although they are produced by the adult worms, Joe (1962) describes "The allergic response is apparently associated with massive destruction of microfilariae, which are continuously trapped and killed in the regional lymph glands before reaching the blood or in different organs such as lungs, liver and spleen after they have arrived in the circulatory system". Further, he suggests that massive destruction of microfilariae in the tissues causes histologic changes, the adult worms being of no importance.

The resistance reported here, while appearing in a local area, is apparently systemic in nature since the injection of blood or pleural washings containing microfilariae from actively infected rats into rats with latent infection intravenously led to their disappearance faster than in controls. Further, in rats with acquired resistance, the microfilaremia induced on transplantation of adult worms in the peritoneal cavity disappeared quicker as compared to controls on prolonged observation (Table XII). In few rats such transplantation resulted in very few or none microfilariae in the peripheral blood possibly due to their quick removal soon after release by other immune sites. In these rats few microfilariae were found attached to cells supporting the view that acquired resistance is general hypersensitivity to the infection. However, it may be that in previously infected, now resistant animal, the concentration of cells at the site of parasitization is far higher than that in the blood as the injections of large number of microfilariae into the thorax of rats with latent infection resulted in none or very low numbers of microfilariae in the peripheral blood (Table XIV). Further, in reinfection experiments the infective larvae developed to adult worms which produce microfilariae but the microfilariae could not penetrate the thorax due to cellular reactions.

On the other hand, infective larvae introduced directly into the thoracic cavity of rats with latent infection resulted in their trapping with immune cells. However, whether it led to their destruction was not seen in the present study.

From the results of the present study it appears that the destruction of microfilariae is occurring rapidly. Injections of thoracic washings of rats with latent infection containing numerous microfilariae trapped by the immune cells into normal rats intravenously resulted in phenomenal disappearance of microfilariae from the peripheral blood within 1-3 hours. This finding together with the gradual disappearance of microfilariae in rats developing acquired resistance strongly suggest another site which is responsible in the destruction of microfilariae. When the various organs of the rats were searched soon after the disappearance of microfilariae from the peripheral blood, large numbers of microfilariae were found to be concentrated in the lungs and to a certain extent in liver. Further, the observations indicate that the microfilariae are possibly undergoing destruction in the tissues as they could not be found in any of the organs 4-8 hours post-injection. It is not known whether these sensitized microfilariae are regarded as foreign material and killed by the reticuloendothelial system of the host or some other mechanisms are involved in clearance of the microfilariae. Whatever the cause may be, the final

picture is a rapid destruction of microfilariae. Hawking (1954) reported that each female worm of L. carinii produces an average of 15,000 microfilariae per day. Matsuda et al. (1968) found that the number of microfilariae produced per day by a female of L. carinii varied from 17,000-43,000, with the average of 28,000. In the present experiments it is shown that the microfilariae can survive for as many as 3 months (half life) in the blood. Similarly Kershaw (1949a) showed that microfilariae of L. carinii may persist for months in cotton rats. This has been demonstrated by transferring microfilariae into normal rats and determining their survival. D. repens transferred into clean dogs survives from 2 months to 3 years (Gruby & Delafond, 1982; Hawking, 1953), and D. immitis for several weeks (Hinman et al., 1934; Wong, 1964a). In the present study as many as 100 or more females were found in some rats with latent infection and no apparent ill effects have been observed even though such large numbers of microfilariae were undergoing destruction.

The present results reveal, for the first time, mechanisms underlying acquired immunity to L. carinii. The major role of lymphocytes in the immune response to infection prompts speculation that delayed hypersensitivity

is somehow involved. The lymphocytes and their derivatives are the only ones capable of transferring delayed type hypersensitivity (Crowle, 1962; Bloom & Chase, 1967). The ability of cortisone, irradiation and ALS in suppressing the acquired immunity provides further evidence that a hypersensitive state of the delayed type may be the factor involved. Larsh (1967b) believes that this mechanism is of prime importance in the production of host resistance to parasitic infections, as has been shown by the transfer of immunity to T. spiralis in mice with cells from peritoneal exudate (Larsh et al., 1964), and the effect of cortisone and whole-body irradiation in suppressing the immune response (Larsh, 1967a). He discussed the work done by others on T. colubriformis in guinea pigs, A. caninum in dogs and F. hepatica and H. nana in mice and concluded tentatively that delayed hypersensitivity is a consequence of infection with these parasites (Larsh, 1967b). Warren et al. (1967) could transfer delayed hypersensitivity in inbred mice with spleen and lymph node cells taken from mice bisexually infected with S. mansoni while the serum was ineffective. Dineen and Wagland (1966) showed that cells from the mesenteric lymph nodes of inbred guinea pigs, made resistant to T. colubriformis, when injected into non-resistant animals of the same genotype, adoptively immunized the recipients against challenge with 1000 infective larvae 4 days later.

It was reported that the fourth larval stage of the parasite was uniquely susceptible to attack, whereas both earlier and subsequent adult stages were not affected by the cells. Further, resistance could be transferred by spleen cells and cells from the lymph nodes other than the mesenteric nodes which drain the site of infection. However, the resistance transferred was less effective than the cells from the local nodes. Attempts to transfer resistance passively with sera from resistant animals, containing both reaginic and precipitating antibodies, were unsuccessful (Dineen, 1967). Dineen and Wagland (1966) concluded that this shows a close analogy with transplantation immunity and hypersensitivity of the delayed type. Further work of Dineen et al. (1968) showed that lymphocytes preferentially localize at the site of infection when injected from immune to infected animals, come into intimate contact with the parasite in the epithelium and rapidly undergo lysis or 'allergic death'. Lymphocytes are considered to be responsible for the transfer of delayed hypersensitivity (Crowle, 1962; Bloom & Chase, 1967), and are involved in specific immunologic phenomena (Harris et al., 1966; Turk, 1967). The recent findings discussed above together with the present work suggesting destruction of microfilariae by lymphocytes stress the importance of cell-mediated immunity as a defence mechanism against parasitic infections.)

The absence of microfilariae in the peripheral blood could be due to two reasons. The microfilariae may be entering the blood but are trapped in immune sites like lymph nodes, spleen, lungs or bone marrow. However, much evidence as could be collected did not support this hypothesis, e.g. splenectomy or partial pneumonectomy did not result in microfilaremia in rats with latent infection. Further, there was no histological evidence for the presence of microfilariae in bone marrow, lymph glands, spleen, liver, kidney or lungs of these rats. The other possibility which seems to be operating was that the microfilariae were prevented from penetrating the capillaries of the pleura due to cellular reactions.

The data on the infection in newborn rats showed that the newborn rats can be infected with L. carinii, but the course of infection is similar to that in 20-day old rats. It appears from the results (Table XXIV) that the resistance to L. carinii infection cannot be altered by exposing the host at the neonatal stage. However, it might be possible that the antigens of the infective larvae are different from that of the microfilariae and the immune mechanism is stimulated only when the infection has matured and the microfilariae appear in the circulation. Following infection about 40 days are required for the infective larvae to become fully mature worms and microfilariae first appear in the circulation about 2 months

after the infection. Therefore, while the infection was given at the neonatal stage the age of the host was about 11 weeks when they were first exposed to the microfilariae and the delay in coming into contact with the microfilarial antigens may be a factor for the negative results. Probably, it would be worthwhile to see the effect of microfilariae in inducing tolerance in the newborn as it has been observed that the immunological tolerance is specific only to those antigens to which the young animal is exposed (Brent & Gowland, 1963).

Experiments on low protein diet show that microfilaremia in rats on standard and high protein diet totally disappeared in about 9-11 weeks after the worm transplant, whereas there was a prolongation of the period of microfilaremia by about 4 weeks in rats on deficient diet (Table XXII). Apparently, then there is a considerable suppression in the development of the immune response in rats fed on low protein diet. Further, the observations would indicate that acquired resistance in rats maintained on low protein diet develops after the disappearance of microfilariae. The conclusion that prolonged maintenance of rats on a low protein diet results in the prolongation of the period of microfilaremia, is in agreement with most other studies on protein malnutrition. Prolongation of

infection in deficient group is not due to failure in the development of antibodies as precipitins were noted in the sera of these animals by gel diffusion. Gordon (1948) reported that in H. contortus infection sheep fed on high protein diet exhibited "self-cure" more rapidly and showed more resistance to challenge than those on low protein. Other studies such as those of Donaldson and Otto (1946) in rats infected with N. muris and Riedel and Ackert (1950, 1951) on the factors involved in limiting the establishment of worms in Ascaridia infection suggest that dietary protein, especially its quality, is important in maintaining resistance to the parasitic infections. Clarke (1967, 1968) showed that significantly larger number of N. brasiliensis larvae reached the lungs in rats on low protein and glucose filter paper diet than in controls. Increase in susceptibility in rats, shown by the delay in the development of acquired immunity, as revealed by the prolongation of infection in rats on low protein diet, as compared to controls was not a result of the failure in the development of antibodies, but due to a reduced cellular reaction in the early stages

of infection. Asirvadham (1948) reported that a 2 per cent protein diet causes atrophy of the bone marrow, liver and spleen, and a significant reduction in lymphocytes and leucocytes. In the present experiments a reduction in cellular infiltration into the thorax might have accounted for lowering of the acquired immunity and prolongation of the infection.

A certain analogy between rat and human infections with regard to host response to infection seems apparent. The present study produces first experimental evidence for the acquisition of a high degree of resistance to reinfection in rats with latent infection. A similar resistance may thus be acquired in man. There are various reports suggesting that man develops a resistance to superinfection in cases of established elephantiasis (Nelson, 1966). Wharton (1947) suggested a similarity in W. bancrofti infection in man and cotton rats infected with L. carinii. The absence of microfilaremia in elephantiasis might be due to cellular infiltration at the sites that harbour adult worms, such as the lymphatics which block the microfilariae from entering into the circulation. It is interesting to note in this connection that Markell and Kerrest (1965) obtained evidence for inflammatory blockage of the lymphatic vessels in patients with elephantiasis, which prevented passage of microfilariae to the blood stream.

These authors could reverse the condition to some extent by cortisone administration. Similarly, the results of the present study suggest that cortisone effects such a reversal in rats with latent infection. The death of microfilariae in the lymphatics may result in certain pathologic manifestations in the tissue. Local collections of dead microfilariae have been reported by Bahr (1912) in inflammatory effusions, and accumulations of dead microfilariae in lymphatics which have been blocked off (O'Connor & Hulse, 1935).

Further, it is possible that infants exposed to infection in early life may not acquire any immunological unresponsiveness to the infection.

## SUMMARY AND CONCLUSIONS

Antigens of the filarial parasites Litomosoides carinii, Dirofilaria immitis, Dirofilaria repens and an intestinal nematode Ascaris lumbricoides have been extracted with phosphate-buffered saline (pH 7.2). The antigens pattern in the parasites has been analyzed by various immunological techniques such as gel diffusion, immunoelectrophoresis, passive cutaneous anaphylaxis (PCA) and agglutination using sera from rats infected with L. carinii and sera from rabbits immunised with antigens of L. carinii and D. repens suspended in Freund's complete adjuvant.

The host-parasite relation in albino rats infected with L. carinii has been investigated in considerable detail with particular reference to the mechanism of the acquired resistance to the infection. The aspects include active and passive immunization, role of humoral and cellular immunity, agents responsible for the development of acquired immunity, effect of protein malnutrition on infection, nature of the immune response as determined by treatment with cortisone, whole-body irradiation, and antilymphocyte serum and the behaviour of rats with latent infection to reinfection.

Attempts were made to infect albino mice with L. carinii.

The immunological aspects in understanding the host-parasite relation in helminthic infections have been reviewed.

The highlights of the investigation are:

(1) Twelve different antigens have been identified in *L. carinii* extracts by immunoelectrophoresis using immunized rabbit serum.

(2) Albino rats on infection with *L. carinii* produce as many as 7 precipitating antibodies.

(3) Among the rat antibodies 'homologous anaphylactic antibody' and 'reagins' have been identified which give PCA in rats at 4 hours and 72 hours respectively after the passive sensitization of the skin.

(4) No heterologous skin-sensitizing antibody akin to that present in immunized rabbit serum could be detected in infected rat serum.

(5) Common antigens between *L. carinii*, *D. immitis*, *D. repens* and *A. lumbricoides* have been detected. Species-specific antigens have also been found in *L. carinii*.

(6) Antibodies could be detected in infected rats by micro-gel diffusion technique as early as on the 7th day after infection.

(7) The antibody titer increased with the progress in infection and was, in general, proportional to the intensity of infection and persisted throughout the course of infection.

(8) Natural infection of albino mice with *L. carinii* through the mite *Ornithonyssus bacoti* resulted in the development of infective larvae to the adult stage, which were soon encapsulated and no microfilaremia was produced. Direct

transplantation of adult worms into the thoracic cavity led to microfilaremia in these animals which persisted in the blood for 3 months.

(9) In mice acquired resistance to filarial infection could not be induced by transplantation with adult worms.

(10) Attempts to immunize rats with killed worm adjuvanted vaccine were unsuccessful.

(11) There was no marked in vitro effect on the activity of adult worms or microfilariae with sera from rabbits hyperimmunized with adult worm antigen or from rats with latent infection.

(12) Passive immunization of rats with rabbit antiserum or sera from rats with latent infection containing circulating antibodies was ineffective.

(13) Humoral antibodies may not have a significant role in the development of acquired immunity to infection.

(14) Irradiation in the range of 20,000 r - 80,000 r did not affect the immediate survival of larvae or the mite. Irradiated larvae could not develop further in the host and did not induce resistance to challenge with normal infective larvae, although antibodies were produced under the experimental conditions.

(15) In general, in rats with latent infection, transplantation of adult worms into the abdominal cavity led to microfilaremia, which, however, disappeared within a few days.

(16) Transplantation of adult worms into the thoracic cavity of rats with latent infection did not lead to microfilaremia. Similar results were obtained when heparinized blood or saline

washings of the pleura of infected rats containing microfilariae were injected into the thoracic cavity of rats with latent infection.

(17) Cortisone and whole-body irradiation break the acquired immunity in rats with latent infection and prolongs the period of microfilaremia in infected rats, or in rats transplanted with adult worms though the preformed antibodies are not affected.

(18) Antilymphocyte serum (ALS) treatment in vivo breaks the acquired immunity as revealed by the appearance of microfilariae in rats with latent infection and addition in vitro frees the microfilariae from the adhering cells.

(19) Splenectomy and partial pneumonectomy did not result in microfilaremia in rats with latent infection. Splenectomy of rats before infection or at declining stage of infection had no effect on the development of acquired resistance.

(20) Microfilariae in blood or pleural washings of infected rats when injected intravenously into rats with latent infection disappeared within few days (2-20 days), whereas in controls they remained in circulation for several months (half life 120 days).

(21) In rats with latent infection there was cellular infiltration in the thorax consisting of largely lymphocytes and macrophages, and to a certain extent giant cells, eosinophils, and mast cells. These cells, in particular

lymphocytes and macrophages, were found to adher to the microfilariae, immobolized them which eventually resulted in their destruction at the site.

(22) Microfilariae in infected mites, fed on rats with latent infection, developed normally to the infective stage.

(23) The sensitized cells that impart acquired immunity, although systemic in nature, appeared to be more concentrated in the thorax which is the site of infection.

(24) Injections of thoracic washings of rats with latent infection containing microfilariae trapped by the immune cells into normal rats intravenously resulted in phenomenal disappearance of microfilariae from the peripheral blood within 1-3 hours and accumulation largely in the lungs and to a significant extent in the liver. About 8 hours after the injection there was no trace of microfilariae in these tissues suggesting rapid destruction.

(25) Rats with latent infection develop a high degree of resistance to reinfection revealed by the absence of microfilaraemia in such rats after a challenge infection through the vector.

(26) Immunity is specific against microfilariae. It neither acts on adult worms nor inhibits their reproduction. At the same time in mite-induced reinfections of rats with latent infection, the infective larvae developed to the adult

stage but resulted in no microfilaremia as the microfilariae produced were trapped by the cellular exudate in the thorax.
(27) A single transplant with 10-15 female worms was sufficient to induce a good resistance to further challenge.
(28) Live males, worms killed with dry ice immediately before transplant or females exhausted of microfilariae were unable to induce resistance.
(29) Direct role of microfilariae in inducing acquired immunity has been demonstrated. Normal rats repeatedly injected in the thorax with living microfilariae, collected from thoracic cavity of infected rats, induce acquired immunity in the recipients, demonstrated by the disappearance of microfilariae from the peripheral blood and by the absence of microfilariae when these rats were transplanted with adult worms.
(30) Protein malnutrition has a depressing effect on the development of acquired immunity to the filarial infection.
(31) Neonatal infection in rats did not induce immunological unresponsiveness.
(32) The major role of the lymphocytes in the immune response and the breakdown of the acquired immunity by cortisone, ALS and irradiation prompts speculation that delayed hypersensitivity plays a dominant role.
(33) A certain analogy in the rat and human filarial infections with regard to host response to infection is discussed.

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